DAIRY COWS

Nutrition, Fertility and Milk Production



Russell E. Marek
Editor

Animal Science, Issues and Professions



NOVA

ANIMAL SCIENCE, ISSUES AND PROFESSIONS

DAIRY COWS: NUTRITION, FERTILITY AND MILK PRODUCTION

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DAIRY COWS: NUTRITION, FERTILITY AND MILK PRODUCTION

RUSSELL E. MAREK
EDITOR



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PREFACE

The elite milk producing phenotype of the modern dairy cow has adversely affected its health. Diminished udder health has serious implications for milk production, leading to decreases in milk yield, milk quality and increases in somatic cell count. This new book presents current research in the nutrition, fertility and milk production of dairy cows. Topics discussed include mastitis in bovine milk production; oxidative stress and reproductive disorders in dairy cows; the incidence of hypocalcemia and its Ca homeostasis mechanism in periparturient cows and the haemodynamic changes of the superovulated corpus luteum in cattle.

Chapter 1 - The elite milk producing phenotype of the modern dairy cow has adversely affected its health. Diminished udder health has serious implications for milk production, leading to decreases in milk yield, milk quality and increases in somatic cell count (SCC). Increases in SCC indicate mastitis, an inflammation of the mammary gland. Mastitis is a significant production disease and a major source of economic loss on dairy farms. It is estimated that 25-40% of dairy cattle are affected at an average cost of €50-€200 per animal due to therapeutic costs, reduced milk yield, milk wastage, penalties for high SCC and involuntary culling. Current mastitis control methods rely heavily on antibiotics for both therapeutic and prophylactic purposes. This is not only costly, but frequently ineffective in chronic subclinical infections. There are also increasing concerns regarding the overuse of antibiotics in veterinary medicine and the emergence of antimicrobial resistant pathogens. This has led to an increased interest in the development of novel approaches to control and treat mastitis, without negatively impacting on milk production. Alternatives currently under investigation include incorporation of mastitis resistance into modern breedingprogrammes, modifications to farm management practices,

identification of non-antibiotic mastitis treatments and enhancement of immunity in cows. This review will discuss recent developments in the fight against mastitis.

Chapter 2 - Oxidative stress seems to be implicated in the pathogenesis of reproductive diseases of dairy cows and subsequent decrease of animal fertility. A major role in the development of oxidative stress is a negative energy balance (NEB) which often occurs in late pregnancy and early lactation. During the NEB, there are metabolic changes accompanied with an increased production of reactive oxygen species (ROS). Although ROS are unavoidable products of normal metabolic processes and are not always harmful, they can impair health and reproductive performance of dairy cows. Mammalian cells possess a natural anti-oxidative system involving in the removal of reactive oxygen molecules and the repairing of oxidative damage. However, exceeded amount of reactive oxygen molecules may have direct and indirect effect on cow's health. In particular, peroxidation of steroidogenic enzymes and steroid hormones can inactivate their function and directly impair reproduction. Additionally, NEB in the early postpartum period is related to endocrine disorders causing a decrease in LH pulse frequency, a decreased diameter of dominant follicles with low estradiol production and decreased systemic and intra-follicular IGF-I availability. These disorders lead to an increased interval to first estrus, poor oocyte quality and weak estrus expression making the detection of estrus even more difficult. As a consequence, ovarian function is disturbed and reproductive performance is impaired. Reproductive diseases including cystic ovarian follicles, anestrus, retained placenta, endometritis and metritis present a great problem in dairy cow's management. Clear understanding of pathophysiology of negative energy balance and oxidative stress could contribute to better approach to reproductive management of dairy cows avoiding reproductive diseases as much as possible.

Chapter 3 - The production diseases of the dairy cow are manifestation of the cow's inability to cope with the metabolic demands of high milk production. While traditionally regarded as encompassing the significant metabolic disorders of dairy cows (hypocalcemia, hypomagnesemia, and ketosis), the term "production disease" has been broadened to include conditions such as retained placenta, displacement of abomasums and laminitis. Most production diseases occur during the first weeks of lactation. The etiology of these diseases can be traced back to insults that occurred during transition period. Grummer (1995) defined the transition period as 3

weeks pre-partum to 3 weeks after parturition. It is a period marked by changes in endocrine status to accommodate parturition and lactogenesis.

Over the past 20 years, the authors' understanding of 'transition cow' metabolism and its relationship to the pathogenesis of peri-parturient disease has greatly increased. There is now significant interest in the critical role peri-parturient disease plays in dairy farm profitability, and in how the risks of such disease and attendant animal culling can be predicted. The risk of many peri-partum diseases of dairy cows is influenced considerably by the nutritional and metabolic status of the animal and in particular, poor adaptation to negative energy balance, is associated with an increased risk of subsequent disease.

Some routinely measured biochemical analytes can be used to predict the development of production diseases in dairy cows. Specific analytes that are either high or low relative to defined reference or 'cut-point' values before calving or immediately post-partum can predict the risk of specific or collective peri-parturient disease events. It was shown that measurement of nonesterified fatty acids (NEFA), β-hydroxy butyrate (BHBA) and calcium concentrations in the first and second week post-partum may provide useful supplementary information for herd health monitoring and culling risk. Hyperketonemia in the first week of lactation is an important risk factor for the subsequent diagnosis of dispalsed abomasums, clinical ketosis and metritis. Additionally, there was a relationship between the concentrations of NEFA at calving and the incidence of certain periparturient diseases. Researchers detected a greater decrease in serum cholesterol concentration and increase in NEFA concentration during the transition period in cows developed retained placenta.

Whole herd interpretation is best made by calculating a proportion of cows above or below a threshold value.

Chapter 4 - Global demand of dairy products and its recent developmentmay put the world supply at risk. To fulfill growing needs, it is widely accepted that a "Livestock Revolution" will be required worldwide. This trend will have to be carried in developing countries, particularly by targeting small scale farms which represent the main actors in animal products' supply chains. However, intervention in such farms requires specific means which consider their characteristics: low cost laborof limited know-how, land, water and financial resources' scarcity. There is an urgent need to reassess the agricultural strategies and the economic managementin these farms, before implementing adapted intervention policies. In this article, the specific case of dairy cattleproduction in Morocco and its possible upgrading to reach international standards is reviewed. The context of cattle farming in

this country is presented first. Indeed, there is a fragmented offer with numerous batches of relatively limited volumes delivered every day. This has induced the emergence of milk collection co-operatives. Milk deliveries are thus organized in a two stage way (from farms to collection centers, then to milk plants), which constitutes a significant constraint to improve quality. Second, intervention possibilities in such chains are presented. A research programwas designed to enhance milk yield and quality. Itsprimary objective was to achieve a diagnosis of dairy production performances (milk yield, raw margin per cow, etc.) in a sample of representative farms. It also allowed characterizing water productivity through dual purpose herds (both milk and meat), as water scarcity represents a priority issue in the agenda of dairying in Morocco. After the diagnosis, an intervention program was implemented by a targeted follow-up of cows' dietary rations and the use of adequate feed supplementation. Results showed that on-farm intervention by balanced rations calculations provided a sound example to assist small scale farmers improve their performances. Finally, models of milk quality parameters in relation to herds' management practices were conceived. They would allow designing a grid of milk quality payment by an indirect assessment of rearing practices. Such results have yet to be adopted at a large scale by the stakeholders in the dairy chain. That implies generalizing the use of such intervention methods, which may necessitate further negotiations devoted to value chain. This may represent practical solutions to upgrade milk production in Morocco, given its numerous contributors and their constraints.

Chapter 5 - The goal of this chapter was to estimate genetic parameters for clinical mastitis (CM) and somatic cell count (SCC) in the first three lactations of Tunisian Holstein cows in order to define how to include this trait as a selection criterion. Mastitis, an inflammatory disease of the mammary gland generally caused by intramammary infections, is the most frequently occurring disease in Tunisian dairy farms. Hence, reduced milk yield, milk quality, and lactation persistency as well as early culling contribute to the economic losses associated with this disease. Mastitis problems were assumed to decrease profitability of dairy cows through milk price, treatment and involuntary culling costs. Somatic cell count (SCC) and clinical mastitis (CM) were analyzed with mixed linear model using data from the first three lactations of 7120 Tunisian Holstein cows having their first calving between 1996 and 2003. Somatic cell counts were log-transformed to somatic cell scores (SCS). The model included fixed effects of year-month and age at calving, and random effects of herd-year at calving and sire. SCC in milk increased as parity increased. The heritability estimates range from 0.009 to

0.12 and from 0.01 to 0.03 for SCC and CM, respectively. The higher genetic correlation between SCC and CM (average 0.65) imply that SCC is an appropriate indicator of the infectious status of the mammary gland. All genetic correlations between CM and SCS were positive, implying that genetic selection on lower SCC will reduce CM-incidence.

Chapter 6 - The objective of this study was to understand status of hypocalcaemia and Ca homeostasis of the local dairy cows during transition period. Sixty multiparous Holstein cows from three intensive dairy farms (I, II, and III, 20 cows per farm) of Heilongjiang province in China were randomly assigned to this experiment in transition period. Their dietary cation-anion difference (DCAD) was in turn 91 meqkg-1 of DM, 152 meqkg-1 of DM, and 85 meqkg-1 of DM, respectively. Concentrations of plasma Ca, hydroxyproline (HYP) 1,25-dihydroxyvitamin D (DHVD), and parathyroid hormone (PTH) were determined at d 21, 14, and7before expected calving, at calving, and at d 7, 14, and 21 after calving. In three farms, the incidence of hypocalcaemia increased near time of calving, reached to the highest at calving (>75%) and then decreased after calving, and plasma Ca was just opposite to it. Compared to other farms, cows in farm II fed a greater positive DCAD had a higher incidence of hypocalcaemia, a lower concentration of plasma Ca and HYP at calving ($P < 0.05$), which indicates that high DCAD inhibited bone Ca mobilization. In addition, cows fed a high positive DCAD in farm II had a slight increase of plasma DHVD concentration ($P > 0.05$) and a higher concentration of plasma PTH at calving ($P < 0.05$), which implicates target tissues were refractory to PTH stimulation. These data demonstrated that hypocalcaemia is very popular during transition period in local three dairy farms. The high DCAD is a major risk factor for hypocalcaemia, which may reduces ability of the cow to maintain Ca homeostasis.

Chapter 7 - The aim of this study was to explore the real time changes in the vascularity of growing superovulated corpus luteum and to compare it with the cyclic CL. Eighteen Holstein-Friesian cows were classified into 2 groups. Group 1; cyclic CL (CCL) in which 7 animal were leaved to get naturally ovulated while group 2; superovulated CL (SCL) (n=11) received PGF2α after 10 days of spontaneous ovulation. After 36 hours, all follicles larger than 5 mm were aspirated at day 0 (D0). The animals were given 28 A.U FSH 24h after aspiration and for 4 days (twice daily, 12h interval). On day 5, the animals received the GnRH analogue. The growth of the CL was examined by Doppler ultrasonography to detect the changes in the blood vasculature in different stages. Blood samples were collected on daily basis and were used to detect Progesterone (P4) using enzyme immune assay (EIA). The results

showed that the superovulated CL was significantly smaller than the cyclic CL but the blood area percentage were significantly ($P<0.05$) higher in (SCL) than the (CCL). It also showed that the P4 level was associated with the vascular activity rather than the size of the Cl.

In conclusion, the superovulated CL has a smaller size, shorter lifespan and less P4 production although the high activity of the blood vasculature in comparison to the cyclic CL.

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Chapter 1

MASTITIS AND BOVINE MILK PRODUCTION

Christine Beecher[1,2], Tommie V. McCarthy[2] and Linda Giblin[1,*]

[1]Teagasc, Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland
[2]Biochemistry Department, UniversityCollegeCork, Cork, Ireland

ABSTRACT

The elite milk producing phenotype of the modern dairy cow has adversely affected its health. Diminished udder health has serious implications for milk production, leading to decreases in milk yield, milk quality and increases in somatic cell count (SCC). Increases in SCC indicate mastitis, an inflammation of the mammary gland. Mastitis is a significant production disease and a major source of economic loss on dairy farms. It is estimated that 25-40% of dairy cattle are affected at an average cost of €50-€200 per animal due to therapeutic costs, reduced milk yield, milk wastage, penalties for high SCC and involuntary culling. Current mastitis control methods rely heavily on antibiotics for both therapeutic and prophylactic purposes. This is not only costly, but frequently ineffective in chronic subclinical infections. There are also increasing concerns regarding the overuse of antibiotics in veterinary medicine and the emergence of antimicrobial resistant pathogens. This has led to an increased interest in the development of novel approaches to control and treat mastitis, without negatively impacting on milk production. Alternatives currently under investigation include

* Corresponding Author.

incorporation of mastitis resistance into modern breeding programmes, modifications to farm management practices, identification of non-antibiotic mastitis treatments and enhancement of immunity in cows. This review will discuss recent developments in the fight against mastitis.

INTRODUCTION – MASTITIS COSTS

In 2007, world dairy production reached 655 million tonnes which included 551 million tonnes of cows' milk [1]. The largest milk producers, by 2006, were Europe (142 million tonnes, 25.8%) and the US (82.6 tonnes, 15%), followed by India, China, Russia, Brazil, New Zealand, Ukraine, Mexico, Argentina and Australia. Milk production has increased dramatically with a 17.2% increase in global production from 1997 to 2007 [1].

However the elite milk producing phenotype of the modern dairy cow is at the expense of health,with negative correlations between mammary health and milk production [2]. Mastitis is considered the most frequent and most costly production disease on dairy farms. It is defined as an inflammation of the mammary gland, caused primarily by microorgansims which invade the udder. The disease accounts for 38% of health related costs on dairy farms [3]. The incidence of bovine mastitis in the UK is estimated at 40 cases per 100 cows [4]. In Ireland, the incidence of mastitis in this grass-based system is approximately 25% [5]. Mastitis indicators suggests this is on the rise [6].

The economic losses caused by mastitis control and management can be divided into losses (revenues not earned) and real expenditures. The most significant economic loss associated with mastitis is a decrease in milk production. The disease causes a 40 to 50% reduction in the economic net margin per cow with the largest part of this loss due to a 5 to 7% decrease in milk yield per lactation [7]. Mastitis is estimated to result in a 100-500kg milk yield loss per cow, per lactation [8-10]. Discarded milk also contributes to revenue losses [11]. In the US, the mastitis cost to the dairy industry runs to $1.2-1.7 billon per year or approximately 6% of the value of production [12].

Expenditure costs include therapeutic, veterinary, culling and replacement costs. The costs of therapeutics arising from treatment and prevention of mastitis can vary depending on mode of delivery, a country's price structure and legislation [11]. A US study by Bar et al. [13] determined the average cost of mastitis in high yielding dairy cows at $179. A study by Kossaibati and Esslemont [3] estimated the cost of veterinary and drug treatment costs, in the UK in 1997, at £60 for mild clinical cases and £90 for severe cases. In Ireland,

costs in 2005 ranged from €45.31- €185 depending on severity of disease [5].The increased risk of being culled was apparent for up to 2 months following a mastitis case [13] and the cost of this higher mortality risk was estimated at $14 per mastitis case [13].Mastitis infected cows shed pathogenic bacteria and therefore pose a potential risk in the transmission of disease [14,15]. In addition, an association between mastitis and other production diseases, such as dystocia and lameness, has been suggested [16,17].

SYMPTOMS OF MASTITIS

Mastitis infections can be classified as either clinical or subclinical. Clinical mastitis occurs where there are visible signs of disease. Mild symptoms of a clinical infection include flakes or clots in the milk (Figure 1) and a slight swelling of the infected udder quarter [18]. More severe signs of infection include abnormal secretion, hot swollen quarter or udder. Cows may have a fever, a rapid pulse, loss of appetite, and suffer dehydration. In extreme cases, death of the animal may occur [19]. Subclinical mastitis events are more difficult to detect as there are no visible signs of disease and no visible changes in milk composition [20]. However, subclinical infections reduce milk yield by 0.3 to 1.8 litres/cow/day and therefore represent the greatest cost associated with mastitis incidence [8,21,22]. Subclinical detection is based on (a) bacteriological culturing of milkor (b) an elevated somatic cell count (SCC) in milk [18]. An elevated SCC is cause by an influx of polymorphonuclear (PMN) cells to the site of infection which constitute the primary defence role in innate immunity against mastitis. In response to recognition of bacteria, several inflammatory mediators induce neutrophil migration from the blood into the mammary tissue. Neutrophils eliminate the invading pathogen by phagocytosis, and respiratory burst [23]. Lymphocytes also play an important role in defence with a shift in trafficking of subpopulations between blood and mammary gland during a pathogen assault. Milk SCC is a key measure of milk quality, reflecting the health status of the mammary gland [24] It is also the key component of national and international regulation for milk quality, udder health and the prevalence of clinical and subclinical mastitis in dairy herds [22]. SCC can be measured using crude but rapid methods, such as the California Mastitis Test (CMT), or more accurate but laboratory based methods, such as automated rapid cell counters.



Figure 1. Healthy and Mastitic Milk.

The degree of infection is dependent on three major factors; the host, the infectious agent and the environment. A specific mastitis event can take place when the bacteria inhabiting the teat canal, invade the udder and colonise. A study examining the diversity of bacteria in the teat canal of lactating dairy cows found 80 different taxonomic groups, of which 20 were from the staphylococci family[25].

BACTERIAL MASTITIS PATHOGENS

Bacterial mastitis pathogens have been loosely classified as either environmental or contagious pathogens. Contagious mastitis pathogens are classed as organisms adapted to survive within the host, in particular within the mammary gland. They are capable of establishing subclinical infections and are typically spread from cow to cow during milking (through milking procedures, contaminated machines and the hands of milkers). They include Gram-positive bacteria*Staphylococcusaureus*, *Streptococcusdysgalactiae*, *Streptococcusagalactiae*, coagulase negative staphylococci (CNS) and *Corynebacterium*spp. Environmental pathogens do not normally infect the mammary gland but can do so when the cow's environment, the teats and udder (or injuries thereof), or the milking machine is contaminated with these organisms and they gains access to the teat cistern. Clean bedding, clean housing, and efficient farm management can control environmental pathogens.Environmental pathogens include the coliforms, *Escherichia*, *Klebsiella* and *Enterobacter*, other gram negative bacteria such as *Serratia*,

Pseudomonas and *Proteus* spp, as well as the Gram-positive bacterium *Streptococcusuberis*

The major pathogens responsible for mastitis infections are listed in Table 1. In the US, Canada and England, coliforms appear to dominate [26-29], while *S. aureus* is highly prevalent in Sweden [30] and Ireland [31]. *Streptococcusuberis* is common in England and Ireland. CNS are emerging as major mastitis pathogens in Germany and the Netherlands (Table 1). Schukken et al.[32] reported that the most expensive mastitis cases, in primiparous cows, resulted from *S. aureus*, *Escherichia. coli* and *Klebsiella* infections, whilst in multiparous cows, *Streptococcus* spp and *Arcanobacteriumpyogenes* infections were also costly. It is worth noting that Gram-negative cases usually result in greater milk losses that Gram-positive cases, with reports of 178kg milk yield differences within 50 days of diagnosis, in a US study [32]. However, in Denmark, the estimated cost of a *S. aureus* infection was €570, *S. dysgalactiae* infection was €149 and *E. coli* infection was €206 [33].

Contagious Pathogens:*Staphylococcusaureus*

The best known and most common contagious pathogen is *S. aureus*, a Gram-positive, catalase and coagulase negative coccus. Once established within the udder, *S. aureus* is difficult to eliminate, as the pathogen can evade the immune surveillance by internalising intracellularly within mammary host cells [34,35]. Antibiotic therapy is often therefore ineffective and dependent on age and lactation stage of the infected animal[36]. Though some *S. aureus*cases may flare up with clinical signs, particularly after calving, the infection is usually subclinical, with no detectable changes in the udder or milk apart from high SCC. Real-time quantitative PCR assays and bacteriological culturing shows that infected cows intermittently shed high numbers of *S. aureus* [37,38], indicating segregation from the herd is important to limit spread of the disease.

*S. aureus*produces over 20 virulence factors that contribute to establishing and maintaining infection [39]. These factors include surface associated proteins and degradative enzymes, such as endotoxins. Surface proteins, namely fibrinogen-, fibronectin-, and collagen-binding proteins, promote colonisation by being able to bind to the host cellular matrix, aiding the internalisation process [40]. Lammers et al. [41] demonstrated that strains deficient in fibronectin-binding proteins were unable to clump and had reduced levels of adhesion and invasion of bovine mammary cells.

Table 1. Incidence of major pathogens (in percent) associated with mastitis in different countries

Country	Ireland	Germany	England and Wales	England	Sweden	Netherlands	US	Canada
Reference	Barrett et al., 2005	Tenhagen et al., 2009	Bradley et al., 2007	Breen et al., 2009	Persson Waller et al., 2009	Sampimon et al., 2009	Grohn et al., 2005	Olde Riekerink et al., 2008
Number of samples	285	751[a], 574[b]	480[c], 464[d]	640[c]	987[c, e]	553[f], 519[g]	493	3033
No growth (%)	27	21[a], 20[b]	26.5[c], 39[d]	31.4[c]	10[c, e]	20.8[f],45.7[g]	18.3	43.9
Coliforms[h] (%)	3	10[a], 13[b]	20[c], 3[d]	27.7[c]	17[c, e]	nd	26.8	12.7
S. aureus (%)	21	10[a], 12[b]	8[c], 10[d]	4.5[c]	21[c, e]	15[f], 3.7[g]	11.6	10.3
Coagulase negative staphylococci (CNS) (%)	9	27[a], 16[b]	8[c], 15[d]	2.7[c]	6[c, e]	38.5[f], 30.1[g]	5.7	5.1
S. uberis (%)	19	9[a], 16[b]	23.5[c], 14[d]	18.9[c]	11[c, e]	7.6[f], 2.1[g]	nd	6.3
S. dysgalactiae(%)	2	14[a], 6[b]	1.5[c], 0.4[d]	0.3[c]	16.5[c, e]	10.9[f], 2.3[g]	nd	4
Streptococci spp. (%)	nd	10[a], 17[b]	nd	0.3[c]	2[c, e]	4.7[f], 3.1[g]	18.5	2.2
Corynebacteria (%)	nd	nd	3.5[c], 10[d]	2.3[c]	nd	11.9[f], 5.2[g]	nd	0.2
Others (%)	nd	16[a], 13[b]	nd	5.4[c]	7[c, e]	17.9[f], 16[g]	nd	12.1

[a] Isolates from samples representing primiparous cows

[b] Isolates from samples representing multiparous cows

[c] Isolates from samples representing clinical mastitis

[d] Isolates from samples representing subclinical mastitis

[e] Estimated values, combining primiparous and multiparous cows

[f] Isolates from samples representing milk SCC >250,000 cells/ml (High SCC)

[g] Isolates from samples representing milk SCC <150,000 cells/ml (Low SCC)

[h] Includes *E. coli* isolates

nd = not determined.

In addition, the *S. aureus* extracellular proteins (coagulase, extracellular fibrinogen protein, extracellular adherence protein and extracellular matrix-binding protein)can inhibit the activation of complement system, by blocking (a) deposition of the complement activating factor C3 on the surface of *S. aureus* and (b) neutrophil binding [42-44]. Following successful colonisation, *S. aureus* bacteria are able to secrete a range of other factors through which they can obtain nutrients, invade, survive and disseminate. The toxins, hemolysins and leukocidins, produced by *S. aureus* can damage host cells through their cytolytic effects [45]. Specifically, leukocidins have been shown to be cytotoxic for bovine erythrocytes and leukocytes [46]. Staphylokinase, produced by *S. aureus* has the ability to bind defensins and inhibit their bactericidal activity [47], while production of enterotoxins by *S. aureus*act as superantigens inducing the production of bovine immune proteins, which downregulate the inflammatory response [48,49].

The emergence of antibiotic resistant *S. aureus* mastitis isolates has become a public concern in recent years. Throughout Europe, penicillin resistance was found in > 10% of strains [50]. Furthermore, methicillin resistant *S. aureus* (MRSA) isolates from bovine mastitis cases have also been identified, with strain typing of MRSA isolates from cattle and humans suggesting the possibility of zoonotic transmission [51,52].

The bovine immune response to intramammary attack with *S. aureus* has yielded conflicting results and highlights the ability of S. aureus to evade the host immune system.This bovine innate immune response is crucial for effective clearance of the pathogen. It is the predominant defence strategy during the early stages of an infection, since it targets a wide range of microorganisms, is ubiquitous and short acting [53]. Toll-like receptors (TLRs)are pathogen-recognition receptorsthat trigger transcriptional activation of pro-inflammatory cytokines and chemokines[54]. The proinflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), once expressed,are potent inducers of the acute phase response. IL-8 is produced by stimulated monocytes, endothelial cells and macrophages among others and attracts neutrophils, basophils and T-cells to the site of inflammation to kill bacteria [55]. Other innate immune proteins of interest include IL-6, which regulates acute phase protein synthesis and promotes monocyte influx into the mammary gland. Interferon-γ (IFN-γ) functions to activate T-lymphocytes, mediate neutrophil activation and induce IL-12 production which, in turn regulates T-lymphocyte differentiation and enhances the cytotoxic activity of natural killer cells and T-cells [42].

Deliberate challenge with a *S. aureus* strain, isolated from a naturally occurring mastitis event, was associated with an absence of clinical signs and no detectable increase in protein levels of, TNF-α, IL-1β and IL-8[56]. Bannerman et al. [57] also reported no increase in IL-8 or TNF-αprotein abundance in response to deliberate challenge. However increases in IL-1β protein levels were reported after 32 h, along with IL-12 and IFN-γ. A slight transient increase in IL-10 was also observed. Alluwaimi et al.[58] measured transcript abundance following *S. aureus* infection. mRNA levels of TNF-α, IL-6 and IL-12 decreased sharply at 7 h pi, followed by a peak 24h pi. However cytokine abundance had reduced again by 32h pi. This cyclic increase and decrease was suggested to mirror the cyclic shedding of *S. aureus* observed during infections. This is in agreement with a study by Riollet et al.[59], where increases in mRNA abundance of *IL-1α*, *IL-1β*, *IL-6*, *TNF-α*, *IL-10* and *IL-12* detected in cells derived from infected glands were observed. In contrast, Lee et al.[60] reported no significant increase in pro-inflammatory transcript abundance, with the exception of *IL-8*, at 32h pi.

Streptococcus dysgalactiae

S.dysgalactiae, a Gram-positive, Lancefield group C bacterium, accounts for a notable proportion of both clinical and subclinical infections [61,62], and exhibits characteristics of both a contagious and an environmental pathogen. It is regularly isolated from infected mammary glands and transmitted during milking [63]. However, extramammary reservoirs of *S. dysgalactiae*, such as cattle tonsils, mouth and vagina have also been identified [64]. Due to the adaptability of this pathogen, traditional mastitis control programmes have had minimal effect on reducing the incidence of *S. dysgalactiae*[65]. Most infections due to *S. dysgalactiae* occur during the dry period or immediately after calving[66]. Different strains of *S. dysgalactiae*, isolated from bovine intramammary infections (IMIs) exhibit variations in intramammary infectivity [67] and express a range of surface receptors that bind to host proteins such as immunoglobulin G (IgG), alpha$_2$-macroglobulin (α_2-M), albumin, fibronectin, fibrinogen, collagen, vitronectin and plasminogen[68]. In addition the bacterium has also been shown to produce extracellular factors that promote establishment and persistence of IMI, such as fibrinolysin, hyaluronidase [69] and streptokinases [70]. *In vitro*, *S. dysgalactiae* has demonstrated the ability to adhere to [68] and internalise [68,71] within mammary epithelial cells. Once internalised, the bacterium is capable of

survival within the host cell [71], without impacting on host cell viabilityas measured by intracellular esterase activity, mitochondrial dehydrogenase activity and plasma membrane integrity [72]. This ability to adhere and internalise no doubt confers protection to the bacteria from antimicrobial drugs and the immune components of the mammary gland. The bovine immune response to *S. dysgalactiae* has been characterised, with an increase in milk SCC evident within 24 h of deliberate challenge [C. Beecher, unpublished data]. mRNA levels of *IL-1β*, *IL-12* and chemokines receptor*CXCR1* increased approximately 40-fold within 48 hours of infusion. These increases were also accompanied temporally with an increase in the mRNA levels of the anti-inflammatory cytokine *IL-10*. The innate immune response to *S. dysgalactiae* was also characterised by an attenuated *IL-8* response with no significant increase observed until 72 h after challenge [C. Beecher, unpublished data]. The tight control of *IL-12* gene expression, coupled with the attenuated *IL-8* response may provide an immune evasion mechanism opportunity for this intracellular pathogen.

Coagulase Negative Staphylococci

More than 10 species different CNS species have been isolated from mastitic bovine milk samples; with the species most commonly reported *S. simulans* and *S. chromogenes*, *S. hyicus* and *S. epidermidis* [73-76]. In general, when isolated from mastitis cases, CNS are not identified to species level, but treated as a uniform group [77]. CNShave traditionally been classified as minor pathogens, due to the generally mild and subclinical nature of IMI [77,78]. However, in some countries, such as Germany and Finland [79,80], they have become the most prevalent mastitis causing pathogen.CNS are considered normal skin microbiota, isolated regularly from cows' teat canals [81] and other extramammary sites such as the vagina, hair coat and nares [82]. An increase in SCC of the infected quarter is usually observed [75]. In clinical cases, local mild clinical signs, such as a slight swelling and changes in milk appearance can be observed [78,83]. *In vitro*, CNS have shown adhesive capacity to mammary epithelial cells comparable to mastitis-associated *S. aureus* strains, although their invasive potential is weaker [84,85]. CNS isolated from caprine mastitis have also been shown to produce at hemolysin, DNAse and elastase [86] . Biofilm associated proteins have also been identified in CNS, with *S. epidermidis* strains testing phenotypically positive for biofilm production [87]. Mastitis events associated with CNS are

still relatively easy to treat with antimicrobials [77,88], with cure rates ranging from 80-90% [77]. An experimental infection was developed to study host response to a deliberate intramammary challenge with *S. chromogenes* [89]. Following challenge, all animals became infected, with mild clinical signs. Milk production of the challenged quarter was decreased for 7 days post infusion. SCC in infused quarters peaked at 30 h post infusion, while *N*-Acetyl-β-D-glucosaminidase (NAGase) activity was highest from 22-46 h post infusion. Milk amyloid A (MAA) and serum amyloid A (SAA) concentrations peaked at 54 and 46 h post infusion respectively [89].

Streptococcus agalactiae

*S. agalactia*e, a Lancefield group B Gram-positive bacterium, is a highly contagious mastitis pathogen within a dairy herd. It is an obligate parasite of the mammary gland and can survive within the mammary gland for extended periods of time [90], causing mainly low grade persistent infections with a low self cure rate [90,91]. Unidentified carrier cows are the main source of infection and are spread by inefficient milking procedures, including failure to administer post-milking teat dipping and dry cow therapy [90,92]. However, efficient on-farm control programs have all but eradicated*S. agalactiae*in Western dairy systems [93]. In addition, antibiotic therapy is generally effective against *S. agalactiae* mammary infection, with amoxicillin and erythromycin particularly effective (86% and 81% cure rate respectively) [94].

Intramammary infusion of *S. agalactiae*, in a mouse model, indicated both local and systemic infection with increases in *S. agalactiae* specific IgA and IgG antibodies observed in mammary glands within 10 days of infection. In addition, serum levels of *S. agalactiae* specific IgG antibodies were also elevated [91]. Neutrophil infiltration, as measured by flow cytometry was observed 6h post infusion. This early response was accompanied by marked increases of TNF-α, IL-6 and IL-1β protein (as measured by ELISA) in mammary glands [91]. IL-10 and IL-12 levels peaked at 72h post infusion [91].

Other Contagious Pathogens

Other contagious mastitis pathogens include *Corynebacterium spp*, including *C. bovis*. These microorganisms are aerobic or facultative anaerobic,

non spore-forming, gram-positive rods. The source of these pathogens is infected udders and is usually spread from cow to cow at milking. They are considered highly contagious pathogens that can cause IMI with a slight increase in milk SCC and a small decrease in milk production in affected cows [National Mastitis Council, 2004].

Environmental Pathogens: *Escherichia coli*

The best studied environmental pathogen is *E. coli*, a Gram-negative rod-like, lactose-fermenting microorganism. This bacterium is present in massive numbers in faeces and is ubiquitous in the cow's environment. As an opportunistic pathogen, the incidence and severity of septic *E. coli* mastitis in dairy cattle is mainly dependent on cow factors, with high-producing cows prone to infectionat parturition and during early lactation. Infection at this time can often result in systemic symptoms, such as diarrhoea, inhibition of rumen motility, paralysis, hypothermia, high pulse rate and hypersalivation [96]. In contrast, infections during mid and late lactation tend to be mild or moderate. Indeed the severity of symptoms determines the degree of production loss and the prognosis for the cow. The lipopolysaccharide (LPS) component of its cell wall is the major virulence factor associated with the pathogen. Indeed intramammary administration of LPS induces the same local signs of inflammation as observed with*E. coli*, albeit it fails to induce the same systemic response, such as milk loss in uninfected quarters [95]. Unlike *S. aureus*, attachment of *E. coli* to mammary epithelial cellsis not necessary for the pathogenesis of clinical mastitis [96,97], with *E. coli* mastitis strains binding fibronectin inefficiently [98]. In fact *E. coli* is an extracellular pathogen and remains in the lumen of the teat canal and lactiferous sinus [96]. Its ability to replicate incredibly quickly, utilise lactose as a carbon source and survive in near anaerobic conditions make it particularly adapted to causing sudden onset of disease, with severe clinical symptoms [96]. Intramammary challenge with *E. coli* yields very different innate immune response compared to a contagious pathogen like*S. aureus* [57] Increases in IL-8, TNF-α, IFN-γ and complement factor C5a were noted within 16 h post infusion [56,57]. Within 24h, increases in IL-10 and IL-12 were also observed, while increases in IL-1β protein abundance were highly variable [56,57].

Streptococcus uberis

One of the most common environmental mastitis pathogens is *S. uberis*. This pathogen survives in soil, bedding material and faeces [99,100], as well as many sites on the cow, such as the lips, rumen, teat skin and udder [101]. IMIs with *S. uberis* can be subclinical or clinical in nature and can persist for long periods of time in a chronic state [102,103]. In experimental infection models, *S. uberis* has been shown to colonise the bovine mammary gland rapidly, induce neutrophil diapedesis and cause local acute inflammation [104]. Adherence, internalisation and intracellular persistence of *S. uberis* within bovine mammary epithelial cells have been demonstrated *in vitro*[105-107]. *S. uberis* adhesion molecule (SUAM) is encoded by the *sua* gene and is conserved in *S. uberis* strains isolated from geographically diverse strains [108]. SUAM plays a pivotal role in adhesion to bovine mammary epithelial cells, with a particular affinity for the innate immune protein lactoferrin. A study by Patel et al. [109] determined that lactoferrin serves as a molecular bridge between SUAM molecules located on the bacterial cell surface and lactoferrin receptors on the surface of bovine epithelial cells. The bridge is thought to provide a mechanism for internalisation and therefore confers a survival advantage to the pathogen. Indeed, deletion of the *sua* gene reduces the ability of *S. uberis* to adhere and internalise [110]. The sortase anchorage proteins, encoded by the *srtA* gene have also been found to play a role in the pathogenesis of *S. uberis*. Mutant strains deficient in the *srtA* gene were only able to infect the bovine mammary gland in a transient fashion and failed to cause clinical mastitis in an IMI model [104]. In addition, the virulence of mutants that failed to express individual sortase substrate proteins was also attenuated in dairy cows [104].

Following intramammary challenge with *S. uberis*, a sustained decrease in milk production (up to 6 days) coupled with a continual increase in bovine IL-8 and C5a protein from 30 h post infusion was observed. In addition, protein abundance of TNF-α, IL-12 and IFN-γ peaked at 1 week post-challenge [111]. A study by Moyes et al. [112] aimed to determine the metabolic pathways affected in response to intramammary infection with *S. uberis*, using microarray technology. Activated pathways in response to *S. uberis* included IL-6 and IL-10 signalling pathways. In total 2,012 genes were upregulated, with 1,082 involved in immune response e.g. IL-6, TNF, IL-8 and IL-10. In contrast, 1,020 genes were downregulated, with a notable proportion involved in milk fat synthesis [112].

Klebsiella pneumoniae

Klebsiellapneumoniae is a Gram-negative rod classified as an environmental pathogen. Outbreaks have been associated with contaminated bedding materials. *K. pneumoniae*-associated mastitisexhibits similar symptoms to *E. coli* mastitis, with severe cases resulting in toxic shock and death. With its poor response to antibiotic therapy and rapid evolution to toxic shock and death, higher losses are incurred with *Klebsiella* mastitis than *E. coli* mastitis [113]. Paulin-Curlee et al.[114] studied the genetic diversity of *K. pneumoniae* from clinical mastitis cases and found that isolates from the same farm were genetically diverse, as determined by repetitive DNA sequence genotyping PCR, pulse field gel electrophoresis and multilocus sequence typing. In this study more than one genotype was found in a single quarter indicating that mastitis can be caused by a variety of *K. pneumoniae* genotypes [114]. Deliberate intramammary challenge with *Klebsiellapneumoniae* results in significant increases of milk SCC, as well as elevated levels of C5a, IL-8 and TNF-α protein within 16 h of infusion [115]. IL-12, IFN-γ IL-10, LPS-binding protein (LBP) and soluble CD14 (sCD14) protein abundance was also increased within 20 h of infusion [115].

Pseudomonas aeruginosa

P. aeruginosa is another Gram-negative mastitis pathogen frequently associated with clinical mastitis. It is water-borne bacterium and sources of infection include contaminated teat wipes, wash water and dry-off preparations used to clean or treat udders [116]. The pathogens minimal nutritional requirements, its ability to grow in soil and water its resistance to chemical disinfectants makes this pathogen virtually impossible to eradicate from the cow's environment[117,118]. *P. aeruginosa* also possesses pili and flagella for motility and adherence, and a number of virulence factors including LPS [119], endotoxin A, exoenzyme S, elastase [120,121]. It is also capable of forming biofilms [122]. The immune response to *P. aeruginosa* is similar to that of *E. coli*, with sustained elevated levels of pro-inflammatory cytokines (IL-1β, IFN-γ, TBF-α, TGF-β1, TGF-β2, sCD14, LBP, and C5a) as well as transient increases in protein abundance of IL-8, TNF-α, IL-10 and IL-12 [116].

TREATMENT

Antibiotic Therapy

Conventional mastitis treatment involves the administration of antibiotics either parenterally or via the intramammary route. Cure rates for a range of antibiotics, alone or in combination, have been reported and can vary due to a number of factors, most notably the causative pathogen, the severity of infection (clinical v subclinical v chronic), the age and lactation stage of the infected cow, and the pharmokinetics and pharmodynamics of treatment administered. Pyorala and Pyorala [123] identified differences in cure rates between clinically mastitic primiparous and multiparous cowswhen treated with penicillin G, with a 57% cure rate in heifers and 27% in older animals. Wilson et al. [94] compared the efficacy of different antibiotics compared to spontaneous cureswhere no antibiotic was administered. Antibiotic treatment is most effective in treating *S. agalactiae* mastitis. Untreated cases showed a 27% cure rate, while antibiotic treatment had an overall cure rate of 77%. Most antibiotics were effective against the pathogen with amoxicillin and erythromycin particularly effective (86% and 81% cure rates respectively). A similar result was observed with other *Streptococcal* spp. with treated animals achieving 83% cure rate versus a cure rate of 66% in untreated controls, amoxicillin again the most effective [94]. Cure rates for streptococcal mastitis with antibiotic cocktailswas reported as high as ~90% (*S.uberis*,*S. dysgalactiae*, *S. agalactiae*) [124].However *S. aureus* poses a serious problem to the dairy industry as cure rates can very from 4-92% [36]. For example Wilson et al. [94] determined that administration of a range of antibiotic treatments (amoxicillin, erythromycin, cloxacillin, penicillin, etc) did not significantly increase the cure rate compared to untreated controls (49% and 43% respectively). Owens et al. [124] determined that a combination of novobiocin and penicillin could be up to 70% effective against *S. aureus* mastitis if treated early within the infection (i.e. within 2 weeks of infection). This cure rate however drops to 35% when the infection becomes chronic. For CNS pathogens, amoxicillin achieved a significantly higher cure rate (87%) compared to untreated controls (72%) [94]. Apparao et al.[125] investigated the efficacy of cepharin sodium against both clinical and subclinical mastitis infections. For clinical mastitis cases, treatment resulted in an overall cure rate of 84% with a 100% cure rate among CNS mastitis. The lowest efficacy was against streptococci at 74%. Subclinical mastitis cure rates were slightly lower overall (81%). Cure rates for *S. aureus* was 83%, with the lowest cure rate

again against streptococci (71%), albeit spontaneous cure rates were unknown [125]. A follow up study by the same group [126] using pirlimycin hydrochloride found no difference in overall cure rate between treatment and control groups (66% each). However treatment gave higher cure rates in CNS and *S. aureus* mastitis (72% and 33% respectively) compared to control groups (66% and 25% respectively) [126]. The large variation in cure rates, the overuse of antibiotics in the industry, the push to reduce prophylactic use of antibiotics and the emergence of antimicrobial resistant pathogens has led to an increased interest in the development of novel approaches to control and treat mastitis, without negatively impacting on milk production.

Probiotics

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Commensal bacteria, with a broad spectrum of antimicrobial activity, have previously been isolated from healthy bovine udders and suggested as potential anti-mastitis agents [127]. The ability of lactic acid bacteria to treat mastitis has been investigated in two separate field trials [128]. The efficacy of intramammary treatment with *Lactococcus lactis* DPC 3147 in chronic subclinical *S. aureus* mastitis infections was investigated by infusing infested quarters with either 2 ml of an overnight broth culture of *L. lactis* DPC 3147 or a commercial antibiotic treatment containing amoxicillin (n=11 quarters for each treatment). By Day 12, seven of the eleven quarters treated with the live culture were pathogen-free, compared with five of the eleven antibiotic-treated infected quarters.SCC remained relatively unchanged regardless of treatment [128].A follow on trial, comparing treatment with a freeze-dried preparation of *L. lactis* DPC3147 with antibiotic therapy was undertaken.The trial included 50 cases presenting with clinical mastitis. Following a 14-day experimental period, similar bacteriological responses were observed in seven out of 25 live-culture treated quarters and nine out of 25 antibiotic treated quarters. Additionally, 15 of 25 cases treated with the culture and 18 of 25 cases treated with the antibiotic did not exhibit clinical signs of the disease following treatment, indicating that treatment with lactic acid bacteria is as effective as antibiotic treatment for presenting mastitis cases [128].

Intramammary infusion with *L. lactis* DPC 3147 also elicits a significant immune response, as determined by flow cytometryand real-time PCR analysis. Probiotic infused quarters experienced a dramatic 12,000-fold

increase in neutrophils within 24 h of infusion [129], with similar increases in lymphocyte numbers observed. Cell-free supernatant and heat-treated probiotic preparations failed to elicit a similar response. In addition, intramammary infusion of healthy mammary glands with the live lactococcal culture results in a rapid and considerable local innate immune response, with pronounced increases in *IL-1β* and *IL-8* mRNA levels [130]. These results suggest that one of the mechanisms responsible for it therapeutic ability is through the stimulation of the host intramammary immune system. The probiotic does not persist in the udder, with no viable lactococci detected in milk 3 days after infusion [130].

The ingestion of probiotics to treat mastitis has also been investigated, albeit in human studies. Jiminez et al. [131] investigated the efficacy of probiotic treatment on treatment of staphylococcal mastitis in lactating women. Women suffering from mastitis, who ingested a combination of *Lactobacillussalivarius* CECT5713 and *L.gasseri* CECT5714 for 4 weeks, showed exhibited lower mean staphylococcal counts in milk and an absence of clinical signs compared to women who received no probiotic treatment [131]. Similarly, Arroyo et al. [132] reported that women assigned to ingestion of *L. fermentum* CECT5716 or *L. salivarius* CECT5713 for 3 weeks had lower bacterial counts in milk, a more pronounced improvement in clinical symptomsand had lower recurrence of mastitis than those assigned to the antibiotic group [132].

Bacteriocins

Bacteriocins are antibacterial peptides produced by bacteriathat display antimicrobial properties against other bacteria [133]. They can inhibit bacteria of the same species (narrow spectrum) or bacteria of different genera (broad spectrum) [134]. Nisin is a natural antimicrobial peptide of 34 amino acids, produced by *Lactococcuslactis* [135]. This peptide has been shown to be effective in inhibiting a wide range of Gram-positive bacteria, including mastitis pathogens [136-138].As such, nisin has been used as an active ingredient in commercially available products for teat-dipping to prevent mastitis [137]. The use of nisin, in combination with lysostaphin, in the treatment of experimentally induced streptococcal and *S.aureus* mastitis has also been reported. Cao et al. [139] investigated the efficacy of nisin treatment compared to gentamicin treatment in clinically infected mastitis quarters. Infusion of 2.5 million IU of nisin yielded a similar cure rate to a dose of 0.8g

of gentamicin treatment (90.2 v 91.1%), as well as similar bacteriological cure rates, and similar proportions of quarters with SCC < 500,000 cells/ml up to two weeks after treatment. However nisin had two distinct advantages over gentamicin treatment. Of the S. *aureus* isolates recovered from infected quarters, none were resistant to nisin, while 82.5% and 35.3% were resistant to penicillin and gentamicin respectively. In addition, nisin was only detectable in milk 12 h following infusion and at low concentrations [139]. Given the financial implications for antibiotic residues in milk, the application of nisin as a mastitis treatment has huge potential. In a similar study, cows with subclinical mastitis received intramammary infusions of nisin (2.5 million IU)daily for 3 days [140]. Cure rates were compared to animals who received no treatment. Nisin therapy had a bacteriological cure rate of 90.1% for *S. agalactiae*, 50% for *S. aureus*, 58.8% for CNS and 62.5% for all cases. Untreated cows had a spontaneous cure rate of 15.9%. Nisin treatment was also associated with more quarters whose milk SCC was reduced to <500,000 cells/ml [140]. One disadvantage to the application of nisin as a mastitis therapy is its reduced solubility and activity at a biologically neutral pH [136].

Lacticin 3147 is another bacteriocin produced by *L. lactis*, which has a broad spectrum against Gram-positive bacteria including those that invade and infect the bovine udder [136,141]. The antimicrobial activity of this bacteriocin is mediated by the combined action of its two component peptides, LtnA1 and LtnA2 [142] (Figure 2). Incorporation of lacticin 3147 into teat seals improved the protection of animals against IMI by *S. aureus* and *Sdysgalactiae*[143-145]. The use of fermentate containing lacticin 3147 as an ingredient in teat dip was shown to reduce numbers of staphylococci and streptococci on the surface of teats by over 80% compared to undipped controls [146]. Synergistic or additive effects have also been reported when pairing lacticin, nisin and lysostaphin in various combinations [147].

Varella Coelho et al. [148] tested the inhibitory activity of seven bacteriocins produced by *S. aureus* (aureocins A70, A53 and 215FN) and *S.epidermidis* (Pep5, epidermin, epilancin K7 and epicidin 280) against over 200 strains of *S. aureus* and *S. agalactiae*. Epidermin and aureocin A53 showed the highest levels of inhibition (>85% and >67%, respectively). Combination of A70 and A53 demonstrated a synergistic effect, broadening the number of strains inhibited compared to the bacteriocins alone [148]. Pieterse et al. [149] reports the characterisation of a narrow-spectrum bacteriocin, macedocin ST91KM, produced by a subspecies of *S. gallolyticus*. This bacteriocin was shown to be active against mastitic streptococcal *spp*. *S. aureus* and *S. epidermidis* [149]

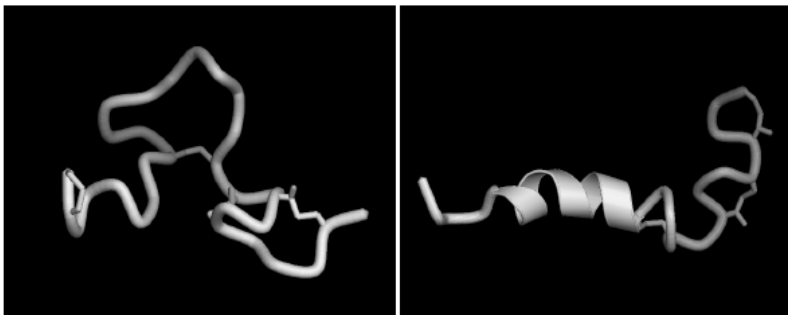


Figure 2. 3-dimensional structure of LtnA1 and LtnA2, the components of Lacticin 3147.

Cytokine Therapy

Cytokine therapy may be a potential candidate for mastitis treatment. The administration of cytokines can result in (1) blocking cytokine production, (2) stimulating the inhibitory pathways (3) removing cytokines from circulation, (4) inhibiting cytokine-binding to receptors and (5) inhibiting signalling mechanisms [150]. IL-1β has been highlighted as a possible treatment adjuvant in S. aureus mastitis. The mature bovine IL-1β protein is 150 amino acids in length with a molecular weight (MW) of 17,732 Da [151]. IL-1β contributes specifically to inflammation by altering vascular permeability, promoting PMN recruitment to the site of infection, inducing vascular endothelial adhesion molecule expression and increasing hepatic synthesis of acute phase proteins [152]. Infusion of 200 μg recombinant bovine IL-1β (rbIL-1β) into mammary glands chronically infected with S. aureus enhances the neutrophil influx in a dose dependent manner and up-regulates inducible oxygen radical formation 150-fold within 16 h of administration [153,154]. However cytokine infusion has no effect on phagocyte efficiency, as determined by flow cytometry analysis [153,154]. Treatment of infected mammary glands with IL-1β increases the cellular potency of certain antibiotics but relapse of the infection can still occur [153,154]. The prophylactic effect of rbIL-1β during the dry period has also been investigated [155]. Intramammary infusion of 10μg rbIL-1β, prior to deliberate challenge with S. uberis, was associated with less new IMIs compared to control quarters (15% and 45% quarters infected respectively). However the potential effect of this therapy was masked by the increase in milk SCC and occurrence of sterile mastitis in cytokine-treated cows [155].

IL-2 has been suggested as a good candidate to boost the immune response of cows prior to or during a pathogen assault [150,153,154]. This interleukin plays a central role in the regulation of adaptive immune response by stimulating T-cells to express cytokines and driving the clonal expansion and differentiation of activated T and B cells [150,156]. The mature bovine IL-2 protein is 135 amino acids in length with a MW of 15,452 Da [157,158]. Infusion of IL-2 in *S. aureus* infected quarters results in recruitment of somatic cells, lymphocytes, neutrophils, macrophages and eosinophils. MHC class II expression is upregulated high antibody titres are observed in milk and serum [150]. A study by Zecconi et al. [159] indicated a low intramammary dose of IL-2 (800pg in 2ml sterile ultrapure water) after calving induced an increase in SCC, SAA, lactoferrin and NAGase, resulting in higher resistance to invading pathogens and a higher number of healthy quarters in the treated group compared to the control group. In addition, this treatment had no side effects on both the cow and milk quality [159]. However serious side effects were also observed with one study associating IL-2 dosage with calf abortions [160]. These results suggest that IL-2 could prove an efficient strategy for mastitis control in dairy cattle, providing administration is controlled in a targeted time and site-specificmanner.

Phage Therapy

Bacteriophages are often highly specific to one or another bacterial species, are non-toxic to animals and plants and usually increase in titer as they infect, multiply in, and kill their target microbes. Bacteriophage K (Phage K), a lytic member of the *myoviridae* family was found to be active against a broad range of *S. aureus* strains [161,162]. However it is unable to replicate in milk contaminated with *S. aureus*. *S. aureus* forms clusters associated with fat globules in raw milk, which was suggested to inhibit phage K adsorption [163]. O Flaherty et al. [164]also reported the isolation and characterisation of two *siphoviridae* phage (SC1 and DW2). These phage were active against bovine mastitis*S. aureus* isolates, and reduced bacterial numbers 10,000 fold alone or in combination, compared to controls. Intramammary infusion of a cocktail of these phage determined that a high titre of phage did not result in an increase in SCC.Their incorporation into teat dips or teat washes as a prophylaxis against *S. aureus* was suggested [164]

PREVENTION AND CONTROL

In the US, studies have estimated the cost of mastitis prevention to vary from as little as €3.50 per cow per year to €24 per cow per year [11]. The susceptibility of cattle to mastitis involves both immune and non-immune mechanisms resulting in the inherent capacity of an animal to succumb to, or resist a mastitis episode when challenged by causative bacteria.Farm management practices, nutrition around the periparturient period, vaccination and breeding have been found to play an important role in mastitis control.

Farm Management Practices

The greatest advancement in the prevention of mastitis was the recommendation of the five-point mastitis control plan in the 1960s [165]. This plan found that implementation of simple hygiene routines, immediate antibiotic therapy of mastitic animals and antibiotic dry cow therapy for every cow in the herd significantly reduced the incidence of mastitis in study herds [166]. The important measures included the following

1. Routine testing of milking machine and equipment
2. Use of post milking teat dipping
3. Prophylactic application of antibiotic therapy for every single lactating animal before the dry period
4. Sampling and treatment of quarters affected by clinical mastitis at the time of detection
5. Culling of cows frequently affected by mastitis during a single lactation.

As a result of the five point plan, the practices of post-milking teat disinfection, and blanket application of antibiotic therapy at drying off became commonplace. The five-point plan has since been further developed to a ten-point plan (Table 2) and places emphasis on maintenance of a dry, clean environment for dairy cows. Implementation of these measures has resulted in substantial falls in SCC [22]. However recently there have been reports of increases in SCC. Berry et al.[6]observed a decline in SCC between the years 1994 and 2000, but from 2000 onwards, a liner increase of more than 2,000 cells/ml/year.

Table 2. Recommended mastitis control measures by the National Mastitis Council, United States[95].

Point	Recommended Control Measure
1	Maintenance of a clean comfortable environment for the cows
2	Use of proper milking procedures such as teat dipping
3	Proper use and maintenance of milking equipment
4	Maintaining detailed records of udder health and milk quality
5	Treatment of clinical mastitis during the lactation
6	Proper management of dry cows including dry-off therapies with antibiotics and/or teat seals
7	Maintenance of biosecurity
8	Culling of chronically infected cows
9	Regular monitoring of udder health
10	Periodically reviewing the udder health control program with a veterinary surgeon

Nutrition

Cows exhibit great susceptibility to a number of diseases during parturition and early lactation. During this period, the dairy cow enters negative energy balance where the energy demands of lactation outweigh energy available from food intake. This severity and duration of the negative energy balance can have a major impact on the cow's immune status [167], with neutrophil function, lymphocyte responses, antibody responses and cytokine production by immune cells impaired[168]. A number of micronutrients have been shown to affect various aspects of immunity in cattle, particularly before and around calving. Trace minerals and vitamins that can influence udder health include vitamin E and selenium (Se), copper, zinc, and vitamin A and carotene.

The amount of concentrate fed to dairy cattle in the period around parturition was identified as a risk factor for veterinary treated clinical mastitis in a Swedish observational study [169]. Cattle consuming stored forages are more likely to be deficient in vitamin E [170].Vitamin E is an integral component of all lipid membranes and plays a role in protecting these membranes from attack by reactive oxygen species [171]. A meta-analysisindicates that increased vitamin E intake by cows can result in a 14% reduction in IMI risk, a 0.7 reduction in milk somatic cell score (SCS, natural log SCC) and a 30% decrease in the risk of clinical mastitis [172]. Cows supplemented with 740 IU of vitamin E per day throughout the dry period had a 37% lower incidence of clinical mastitis during the next lactation compared to cows fed ad libitum haylage which provided approximately 320 IU of vitamin E [173]. Although many studies have investigated supplementation with vitamin E, the combined supplementation of vitamin E and Se has become the more popular choice. Se is an essential micronutrient in tissues throughout the body and is also an integral component of the enzyme glutathione peroxidase [174,175]. A survey of blood Se concentrations in Norwegian Red heifers and dry cows and found that the risk of mastitis was 1.3 to 1.4 times greater in animals deficient in Se (<0.06μg/g wet weight blood)than theircounterparts with whole blood levels of Se above 0.11μg/g [176]. In addition, Weiss et al. [177] found that high serum Se concentrations were associated with reduced rates of clinical mastitis and low bulk tank SCC (BTSCC) but injection of 0.1 mg/Se/kg body weight 21 days before calving had no effect on incidence of clinical mastitis [173].However, prepartum supplementation of both vitamin E and Se decreased incidence, duration and severity of mastitis in dairy cattle [173]. Indeed, cows supplemented with both vitamin E and Se that succumbed to mastitis had shorter duration of clinical signs than mastitic cows supplemented with either micronutrient alone [173]. Supplemented heifers (0.3 ppm Se and 1000 IU/day vitamin E), from 60 days prepartum, had (a) significantly fewer quarters infected at calving, (b) reduced prevalence of infection throughout lactation, (c) fewer cases of clinical mastitis, (d) infections of shorter duration, and (e) lower milk SCC compared to unsupplemented heifers [178]. Following intramammary challenge, this dietary supplementation appears to result in a rapid PMN influx into milk, with increased intracellular killing of ingested bacteria by PMN [179].

Copper (Cu) is a component of ceruloplasmin, which facilitates iron absorption and transport and is also an important part of superoxide dismutase, an enzyme that protects cells from the toxic effects of oxygen metabolites produced by phagocytosis [180]. Research has indicated that dietary Cu affects

phagocytic and specific immune function [181,182]. Heifers fed Cu sulphate at 20 ppm form 60 days pre-calving to 30 d into lactation had less severe symptoms following deliberate intramammary challenge by *E. coli* compared to heifers fed a basal Cu diet (6.5 ppm) [183]. However there was no difference in the length of infection [183]. Scaletti et al. [184] also reported that the dietary source of Cu had an effect, with heifers fed supplemented Cu proteinate (10 ppm) from 60 days pre-partum until 49 days of lactation exhibiting lower bacterial counts and greater dry matter intake (DMI) and milk production, compared to animals supplemented with Cu sulphate (10 ppm) or control animals (6.5ppm)

Zinc is required for keratin formation, skin health and skin integrity. Supplementation with zinc has been associated with reducing SCC in some herds, but this has not been confirmed in other studies [180,185]. Two studies, investigating the effect of replacing inorganic zinc sulphate with an organic form in diets, associated the organic form with a decrease in new IMIs and lower SCC [170].

Vitamin A and it precursor, β-Carotene, are important for the maintenance of epithelial tissue health, and also play roles in mucosal surface integrity and stability [182,186]. Low concentrations of plasma vitamin A and β-Carotene have been associated with severity of mastitis [187]. However, other data indicates that dietary supplementation with these micronutrients(170,000 IU/day vitamin A and 300 mg/day β-Carotene) had no effect on clinical mastitis incidence or SCC through the dry period, calving or 6 weeks postparturition [188]. On the other hand, Dahlquist and Chew [189] reported that animals supplemented with 53,000 IU/day vitamin A and 300mg/day of β-carotene, starting 3 weeks before drying off and continuing through the dry period, had fewer infections in the dry period than cows supplemented with vitamin A alone. Contradictions between these studies can be possibly explained by the differences in plasma β-carotene before commencement of the studies, with plasma β-carotene levels reported by Oldham et al. [188] four times that of mean plasma levels reported by Dahlquist and Chew [189].

Vaccines

The development of mastitis vaccines remains elusive. Vaccines have been found to be most effective in the prevention of coliform mastitis, while vaccines against other pathogens (in particular, *S. aureus*) have shown limited efficacy under field conditions. Administration of the *E. coli* specific J5

vaccine (derived from an *E. coli* mutant strain lacking O-polysaccharide chains within its LPS)eight weeks and four weeks prior to calving, was associated with reduced SCC, faster clearance of *E. coli* from milk, and 75% less milk loss, following intramammary challenge with *E. coli* in early lactation. In addition, vaccinates were significantly associated with higher production of J5-specific IgG1 and IgG2 in early lactation, which are beneficial in phagocytosis by PMN and clearance of bacteria from the mammary gland [190,191]. Indeed, the risk of culling is significantly lower for J5-vaccinates [191]. However,vaccination did not reduce incidence rates of *E. coli* mastitis [190] or prevent infection following deliberate challenge [191], but rather reduced the severity of the clinical symptoms and the degree of production losses associated with *E. coli* mastitis.

The efficacy of a Lysigin, a commercially available vaccine against *S. aureus*, has been investigated in multiple studies.Lysigin is a lysed culture of highly antigenic polyvalent somatic antigens of *S. aureus*. Nickerson et al. [192] vaccinated heifers at 6 months of age, followed by a booster dose 2 weeks later and subsequent vaccinations every 6 months until calving. Vaccinates had a 45% reduction in new *S. aureus* infections during pregnancy and calving relative to controls. In addition vaccinates had a 30% reduction in new CNS IMI. However, studies by Middleton et al. [193,194] and Luby et al. [195] failed to repeat these results, albeit the dosage regime differed between studies. Although Middleton et al. [194] observed no differences in *S. aureus* clearance rates between vaccinates and control animals, the authors did report lower mean duration of clinical mastitis and lower total mastitis score post-challenge than controls. Trials performed with lactating dairy cows demonstrated that two doses of Lysigin during lactationdoes not reduce the incidence of new *S. aureus* or CNS mastitis infections, or alter milk SCC or milk antibody isotype titre compared to unvaccinated animals. However early vaccination, followed by multiple immunisations before calving may help control staphylococcal mastitis in heifers.

Pellegrino et al. [196] investigated the efficacy of a *S. aureus* avirulent vaccine. Pregnant heifers received a subcutaneous dose of the vaccine 30 and 10 days before calving. Subsequently, at 10 days post-calving, vaccinated and control heifers were deliberately challenged with a homologous *S. aureus* virulent strain. A significant increase in IgG specific to the *S. aureus* strain was detected in the blood and milk of vaccinates compared to control heifers, as well as a slight increase in daily milk yield throughout the trial. However, there was no difference in bacterial shedding or SCC [196]. Plasmid DNA, containing *S. aureus*sequences which code for peptidesimportant in the

pathogenesis of the disease exhibit protective effects when used as a vaccine [197].

Bolton et al. [65] describes the use of surface proteins, GapC and Mig, as protective antigens against *S.dysgalactiae* mastitis. Cows entering the drying off period received two subcutaneous vaccinations of either GapC protein or Mig protein at 31 and 10 days prior to a*S. Dysgalactiae* intramammary challenge. Animals vaccinated had significantly lower SCC than control animals in the 3 days immediately after intramammary challenge. In addition, quarters of animals vaccinated with the GapC protein had significantly lower pathogen numbers [65].

Finch et al. [198] investigated the effect of immunization of dairy cows against *S. uberis*. Bacterial surface extract was administered to cows subcutaneously at 14 days before drying off and again within 48 h of calving. Animals were then deliberately challenged with *S. uberis*. Vaccination reduced bacterial shedding, SCC and milk production loss in animals challenged with the homologous strain, but failed to prevent infection with heterologous strains, albeit bacterial colonization was reduced [198]. Leigh et al. [199] aimed to determine the effect of vaccination with preparations containing the *S. uberis*plasminogen activator, PauA. Quarters of cows vaccinated with PauA, and then challenged with *S. uberis* hadlower bacterial numbers in milk [199].

The use of adjuvants to increase the efficacy of vaccines in mastitis prevention has also been investigated. Yin et al. [200] report the cloning of adhesion clumping factor A (ClfA), the main fibronectin binding protein geneof *S. aureus* and bovine IL18 (bIL-18) and their combined use in a murine mastitis model.A recombinant DNA vaccinecontaining bIL-18 alone stimulated a cellular immune response, with secretion of IL-12 and IFN-γ (as determined by ELISA) observed. A recombinant vaccine containing both ClfA and bIL-18was associated with significantly higher secretion of IL-4 and IL-10, as well as increased lymphocyte proliferation, compared to unvaccinated controls [200].

Breeding for Disease Resistance

There is a genetic component to whether or not a cow will succumb to mastitis once under attack by an udder pathogen. Mastitis is a complex trait, with mastitis heritability, ranging from 0.01 to 0.06 [201-203] and SCC heritability at 0.1[204]. The genetic correlation between mastitis and SCC is

high [204-207]. Selection for decreased SCC has been shown to decrease mastitis without weakening natural defences [208,209]. Indeed, sheep bred for low somatic cell score (SCS, natural log SCC) possess a better ability to limit and eliminate mastitis infections that their high SCS counterparts [210]. For the purposes of breeding programmes, SCC has been suggested as a more reliable indicator of udder health than mastitis [211]. Global and candidate gene approaches have been employedin the quest for (a) genomic regions (b) genes and (c) polymorphisms that play a role in mastitis resistance.

In various cattle breeds, over 50 genomic regions have been identified that associate with udder health. These discoveries are reviewed in detail by Khatkar et al. [212] and Ogorevc et al. [213].Quantitative trait loci (QTL) for clinical mastitis have been identified on *Bostaurus* autosomes (BTA) 3, 4, 5, 6, 8, 9, 11, 13, 14, 18, 25, and 27 [214-216]. QTL for SCC have been identified on BTAs 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 13, 14, 15, 18, 19, 20, 21, 22, 23, 26 and 27 [214,215,217-226]. The telomeric end of BTA18 has received considerable attention for udder health. Independent studies, using Swedish Red and White cattle, Finnish Ayrshire cattle, and Holstein herds in the US, Sweden Germany and the Netherlands, have identified a QTL for SCS and/or mastitis at this location, within an eleven centimorgan (cM) range [214,216-218,222,224,227-229]. Heyen et al. [220] also reported a QTL for SCS on BTA23, in close proximity to the bovine major histocompatibility complex (MHC). Klungland et al. [215] detected a QTL on BTA8, adjacent to four interferongene loci. In a review by Ogorevc et al. [213] the chromosomes with the highest density of mastitis related QTL were BTA3 and BTA14. Lund et al. [230] identified markers on chromosomes 6, 9, 11, and 15 for use in marker-assisted selection programmes to reduce clinical mastitis without any deleterious associationswith milk production traits. Sorenson et al. [231] identified pathogen specific QTL on BTA5 and BTA15. A QTL for SCS located on BTA5 associated with *E. coli* and *S. aureus* infections, whereas a QTL on BTA15 associated with *S. aureus* and *S. dysgalactiae* mastitis events [231].

With the development of high density single nucleotide polymorphism (SNP) chips, the application of genome wide association studies (GWAS) is becoming more popular. GWAS is a non-hypothesis driven method that can be used to localise genomic regions that contribute to natural genetic variation in any phenotypic trait. The identified regions can then be fine-mapped at higher marker density to allow the efficient identification of candidate genes [232]. GWAS has advantages over conventional linkage-based QTL mapping, namely greater study power, more precise QTL location and less computertime than conventional methods [233]. However, there are limitations

to GWAS. Large study populations are needed to find significant associations after adjusting for multiple testing and fine mapping is still required after locating regions to identify causative mutations and/or candidate genes for further investigation [234].

The candidate gene approach involves identifying genes involved in immune pathwaysof the mammary gland and locating SNPs which may influence function of the encoded protein. A number of polymorphisms have been identified and associated with mastitis and/or SCC. Bovine genes of interest are those involved in immunity response, particularly pathogen recognition and neutrophil function. For instance, the MHC genes code for immune proteins controlling the induction and regulation of the adaptive immune response [235]. Genes within the complex can be divided into Class I and Class II genes. Class I genes are expressed at the surface of all nucleated cells and interact with cytotoxic T-cells. Significant associations between several alleles in class I genes and mastitis indicators, such as CMT score, clinical mastitis, SCC and antibody response to infection. [236-239]. Expression of class II genes is restricted to antigen presenting cells. Exon two of the class II DRB locus is highly polymorphic and encodes the antigen adhesion site of MHC molecules [235]. Three independent studies have associated allele BRD3.2*24 with susceptibility to mastitis by various pathogens [240-242].

CXCR1 encodes a chemokines receptor on neutrophil surfaces which binds pro-inflammatory cytokine IL-8 with high affinity. The bovine *CXCR1* gene maps to BTA 2 at position 110 megabase-pairs (Mbp) and encodes a protein of 360 amino acids in length [243]. A QTL for SCS is also linked to BTA2 atposition 105 Mbp [213]. A polymorphism (G/C) at position 777 of CXCR1 results in a glutamine to histidine substitution at amino acid position 245 located within the third intracellular loop [243]. Studies by Rambeaud et al. [244,245] reported an association between the CC genotypeand impaired neutrophil migration and reactive oxygen species production *invitro*, and an increase in subclinical mastitis*in vivo*. Others studies have failed to find associations with the CC genotype and mastitis incidence and/or SCC [246,247, C. Beecher, unpublished data].

TLR4 is an innate immune protein on cell surfaces that recognizes lipopolysaccharide (LPS) of Gram-negative bacteria [248]. The TLR4-2021 (C/T) polymorphism, within the third exon, results in the substitution of threonine with isoleucine at amino acid position 674, located within the transmembrane-cytoplasmic domain [249]. The C allele associates with decreased SCS and higher lactation persistency in the daughters of Holstein-

Friesian bulls [250]. This association did not however hold true for Simmental, Holstein and Sahne cattle in a Chinese study [251]. Combined with this, *TLR4* exhibits a differential expression during mastitis episodes [252,253], which prompted Ogorevc et al. [213] to propose *TLR4* as a strong candidate for inclusion in cattle breeding programs to augment the accuracy of selection for mastitis resistance. Wang et al. [251] identified a SNP within the promoter region of the TLR4 gene and associated the A allele with lower SCS than allele B.

Forebrain embryonic zinc finger (FEZL) like protein is a transcription factor with a role in neuronal development. The gene is mapped to a fine region on BTA22, identified by Heyen et al.[220] as having a QTL for mastitis. Mastitis induces FEXL expression in mammary glands and induced FEZL promotes expression of the axon-attracting molecule semaphorin 5A (SEMA5A), which can induce IL-8 and TNF-α expression [254]. A three base insertion results in the addition of an extra glycine (13G) in the protein and this variant associatesincreased susceptibility to mastitis. SEMA5A gene expressionis downregulated in cows carrying the 13G variant [254].

Lactoferrin is a glycosylated protein noted primarily for its iron homeostasis, antibacterial, antiviral, and immune modulatory properties. The bovine lactoferrin gene is mapped to BTA22, contains 17 exons and codes for a protein 708 amino acids in length [255]. It is located in neutrophils and is found in a wide variety of secretions, including colostrum, milk, vaginal fluid, semen, tears, saliva, nasal secretions, bile and gastrointestinal fluid [256]. The lactoferrin gene is highly polymorphic, with multiple SNPs identified within the promoter and coding region [256-260]. A polymorphism (C/T) within the lactoferrin promoter region (LF-586) was recently associated with SCS in Holstein Friesian sires [261]. The T nucleotide at position−586 disrupts a consensus binding sequence for the activator protein 2 (AP2) transcription factor [262]. This sequence disruption may weaken the affinity of AP-2 for the lactoferrin promoter and hence reduce lactoferrin transcription. In addition, the LF-586 SNP also lies within a selenocysteine tRNA gene transcription-activating factor (Staf) binding site. Staf is primarily involved in thebiosynthesis of selenoproteins, which have supportiveroles in mammary gland health [263,264].It is proposed that disruption of Staf binding would reduce lactoferrin expression. Indeed, homozygous TT cows had lower lactoferrin protein concentrationin their milk compared with their CC herdmates [Bahar et al., unpublished data]. Other Lf promoterpolymorphisms (LF32-C/G, LF−478-del, and LF72-T/C) have also shown associations with somatic celldata [258,260]. However, *in silico* analysis did not identify

transcriptional regulation matrices at these locations [265], indicatingthat these SNP may not be causal mutations.

Other potential immune targets for incorporation into breeding programs include polymorphisms in the cluster of differentiation 14 (*CD14*), caspase recruitment domain 15 (*CARD15*) and *SERPINA*genes. *CD14* encodes a membrane-associated protein on the surface of cells and is involved in the recognitionof LPS in conjunction with TLR4 [266]. Of the exonic SNPs identified in the *CD14* gene, CD14-1908 (A/G) causes an asparagine to aspartic acidsubstitution at amino acid position 175 within the leucine rich repeat (LRR) domain involved in pathogen recognition [267]. Animals expressing the mutant variant have a higher percentage of neutrophils expressing CD14 molecules on their surface which may increase the speed of response to pathogen attack [267]. However no association was between this polymorphism and SCS in Irish Holstein bulls [C. Beecher, unpublished data]. CARD15 is a cytosolic protein that initiates inflammation following microbe recognition [268] and plays an important role in nuclear factor-kappa B (NF-κB) activation [269].TheCARD15-3168 (A/T) polymorphism, within exon 12, results in a leucine$_{125}$ to glutamine switch within the LRR domain [270]. Statistical analysis of the genotypes and phenotypes of 338 Canadian bulls revealed an association between the T allele and increased SCS values [270].However, this association was not found within the Irish Holstein Friesian sires[C. Beecher, unpublished data]. *SERPINA1* encodes a serum protease inhibitor, whose primary role is to protect tissues against neutrophil attack. In particular, secretion of this protease protects 'self' cells from neutrophil elastase digestion [271]. A*SERPINA1*haplotype, composed of five SNPs within exon regions (SERPINA1-164, -269, -284, -407, -989), was found to associate with decreased SCS in US Holstein dairy cattle [272], although no association was found in Irish Holstein Friesian sires [C. Beecher, unpublished data].

Transgenic Animals

Transgenic animals have also been identified as a potentialmethod to combat mastitis. By using the promoter and regulatory components of a gene encoding a milk protein, it is now possible to target expression of the transgene of interest in the mammary gland [273-275]. Transgenic mice expressing bovine soluble CD14 (bsCD14) in their milk (31 to 316 μg/ml) exhibited reduced infection-associated edema in response to deliberate *E. coli*

intramammary challenge. However the overexpression of bsCD14 did not enhance early inflammatory cytokine expression of reduce bacterial load in the mammary gland [276]. Lysostaphin, a peptidoglycan hydrolase, is produced naturally by *S. simulans* and cleaves glycyl-glycine bonds within the cell wall of staphylococci, with *S. aureus* particularly sensitive [277,278]. Three transgenic cows, secreting lysostaphin at concentrations ranging from 0.9 to 1.4 mg/ml in their milk when experimentally challenged with an intramammary infusion of *S. aureus* were asymptomatic compared to their nontransgenic counterparts. Lactoferrin is expressed at relatively low levels in healthy mammary glands but increases during IMI, depending on invading pathogen [279]. Transgenic cows expressing recombinant human LF in milk (rhLF; average 2.9 mg/ml) suffered less sever clinical signs and recovered faster from *E. coli* challenge than control animals. However there was no difference in susceptibility to disease [280]. A later study found that transgenic animals expressing rhLF eliminated *S. chromogenes* bacteria faster than control cows and had significantly milder inflammatory response [281]. However there are a number of issues with regard to the use of transgenic animals in preventing mastitis not least of which is public perception and the presence of transgene products in the food chain.

CONCERNS FOR THE INDUSTRY

The ability to deliver high quality milk products from healthy animals is essential for the dairy industry [22]. Mastitis, both clinical and subclinical infections, can have serious deleterious effects on milk quality. Currently the European Union has placed a regulatory limit of 400,000 somatic cells/ml of milk supplied [EEC, 1992, Council Directive 92/46/EEC]. To ensure compliance, milk processors often impose a penalty on milk with high SCC (>400,000SCC/ml) based on a tiered monthly arithmetic mean bulk SCC. Some processors have also introduced bonus schemes for herds achieving 200,000 cells/ml or lower for bulk milk SCC. The European quality limits have been adopted internationally as the standard expected of exports [22], although the USA and Canada have national limits of 750,000 and 500,000 cells/ml respectively [282,283].

Mastitis is associated with increased activity of heat-stable proteases and lipases leading to a breakdown of casein proteins and milk fat [284,285]. Ma et al. [286] also determined that high SCC milk reduces curd firmness, decreases cheese yield, increases fat and casein loss in whey and decreases

sensory quality in the production of cheese. Herds with mastitis are also at risk of antibiotic residue violation [283,287].

There is no evidence that milk with high SCC poses any risk to human health [95]. High cell counts are however associated with indirect risks, including poor farm hygiene, antibiotic residues and the presence of pathogenic organisms and toxins in milk. [95].

CONCLUSION

Aggressive selection for milk production over the years, with little or no emphasis on functional traits [288] has been associated with a decline in udder health [289]. Mastitis has an impact on animal production, animal welfare and the quality of milk produced [290]. Today, the health and welfare of dairy cattle is seen as vital to maximise profitability and to enhance public perception of modern dairy systems [93]. While traditional veterinary medicine focussed on diagnosis of disease and treatment of individual animals, modern veterinary practice recommends a more herd management orientated approach, resulting in a healthy herd.

Current mastitis research is focussed primarily on discovering methods to prevent risk of IMI. Long term breeding goals, incorporating data from association analyses, QTL studies and crossbreeding initiatives will aid in the selection for a more robust animal. In addition the development of effective farm management and vaccine strategies will help to protect against infection. In the search for alternative therapies, the application of probiotics, bacteriocins and cytokine therapy, are promising developments. Extensive *in vivo* animal trials are still required to determine their efficacy inside the farmgate. The application of alternative therapies in combination with antibiotics, particularly in the case of chronic infections, may improve cure rates. Indeed the true benefit of these alternative treatments may be in the growing organic farming sector, where the use of antibiotics is limited.

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Chapter 2

OXIDATIVE STRESS AND REPRODUCTIVE DISORDERS IN DAIRY COWS

Romana Turk[1,*], Marko Samardžija[2] and Goran Bačić[2]
[1]Department of Pathophisiology
[2]Department of Reproduction and Clinic for Obstetrics
Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55,
10000 Zagreb, Croatia

ABSTRACT

Oxidative stress seems to be implicated in the pathogenesis of reproductive diseases of dairy cows and subsequent decrease of animal fertility. A major role in the development of oxidative stress is a negative energy balance (NEB) which often occurs in late pregnancy and early lactation. During the NEB, there are metabolic changes accompanied with an increased production of reactive oxygen species (ROS). Although ROS are unavoidable products of normal metabolic processes and are not always harmful, they can impair health and reproductive performance of dairy cows. Mammalian cells possess a natural anti-oxidative system involving in the removal of reactive oxygen molecules and the repairing of oxidative damage. However, exceeded amount of reactive oxygen molecules may have direct and indirect effect on cow's health. In particular, peroxidation of steroidogenic enzymes and steroid

* Correspondence: Dr Romana Turk, University of Zagreb, Faculty of Veterinary Medicine, Department of Pathophysiology, Heinzelova 55, 10000 Zagreb; email:rturk@vef.hr.

hormonescan inactivate their function and directly impair reproduction. Additionally, NEB in the early postpartum period is related to endocrine disorders causing a decrease in LH pulse frequency, a decreased diameter of dominant follicles with low estradiol production and decreased systemic and intra-follicular IGF-I availability. These disorders lead to an increased interval to first estrus, poor oocyte quality and weak estrus expression making the detection of estrus even more difficult. As a consequence, ovarian function is disturbed and reproductive performance is impaired. Reproductive diseases including cystic ovarian follicles, anestrus, retained placenta, endometritis and metritis present a great problem in dairy cow's management. Clear understanding of pathophysiology of negative energy balance and oxidative stress could contribute to better approach to reproductive management of dairy cows avoiding reproductive diseases as much as possible.

INTRODUCTION

A number of studies suggest the role of oxidative stress in reproductive disorders and low fertility of farm animals (Miller et al., 1993; Harrison et al., 1984; Allison and Laven, 2000). Reactive oxygen species (ROS) are involved in the physiology of cow´s reproductive system and an imbalance between ROS production and antioxidants is supposed to be implicated in pathological processes influencing reproductive disorders (Agarwal et al., 2006). A major role in the development of oxidative stress in cows is a negative energy balance (NEB) which often occurs in late pregnancy and early lactation(Roche et al., 2000). During the transition period in dairy cows with NEB there are metabolic changes and disorders accompanied with an increased production of reactive oxygen species (ROS) (Bell, 1995; Mudron et al., 1999; Herdt, 2000). Although ROS are unavoidable products of normal metabolic processes and are not always harmful, they can impair health and reproductive performance of dairy cows. Mammalian cells possess natural anti-oxidative system, which is, in part, due to activities of various enzymes involving in removal of reactive oxygen molecules and repair of the oxidative damage (Wiese et al., 1995). However, an imbalance between production of ROS (pro-oxidants) and their safe removal by antioxidants is commonly defined as oxidative stress (Lykkesfeldt and Svendsen, 2007). Exceeded amount of reactive oxygen molecules may have direct and indirect effect on cow's health. Directly, ROS can oxidize macromolecules such as lipids, proteins and DNA and cause oxidative cell injury. Indirectly, ROS can damage cellular components and membranes and thus modify metabolic pathways (Miller et al., 1993).

Particularly, peroxidation of steroidogenic enzymes can inactivate their function and directly impair reproduction.

OXIDATIVE STRESS

Reactive Oxygen Species (ROS)

Aerobic organisms constantly face the oxygen (O_2) paradox: although they require O_2 to support life, aerobic respiration and metabolism generate reactive oxygen species (ROS) as their by-products arising from either mitochondrial electron transport chain or from stimulation of NADPH (Valko et al., 2007).Cytocrome P-450 enzymes are an important source of ROS metabolizing either exogenous (xsenobiotic) or endogenous (physiological) substances (Miller et al., 1993).

ROS are physiologically used by cells in intracellular signaling and redox regulation (Nordberg and Arnér, 2001). Physiological level of ROS play an important role in the regulation of reproductive processes such as folliculogenesis, oocyte maturation, corpus luteum, uterine function, embryogenesis, embryonic implantation and feto-placental development (Agarwal et al., 2008). However, an imbalance between ROS production and their safe removing lead to oxidative stress. Exceeded amount of ROS can modify cell functions and endanger cell survival. Thus, they must be inactivated keeping only a small amount of ROS necessary to maintain normal cell function (Agarwal et al., 2003; Al-Gubory et al., in press).

ROS that cause oxidative damage may be divided into two categories: free oxygen radicals and non-radical ROS. Free oxygen radicals could be defined as any chemical species containing one or two unpaired electrons (Shackelford et al., 2000). Although non-radicals do not contain unpaired electrons, they are very unstable and can react with free radicals resulting in a new radical leading to chain reaction of free radical formation (Halliwell and Gutteridge, 1984). Common free oxygen radicals include hydroxyl radical (·OH), superoxide anion ($\cdot O_2^-$) and nitric oxide (·NO) while non-radical include hydrogen peroxide (H_2O_2) (Halliwell et al., 1992). They all are ubiquitous, unstable, highly reactive and diffusible molecules. They become stable by acquiring electrons from lipids, proteins, nucleic acids, carbohydrates or any other nearby molecule leading to chain reactions of free radical molecules formation resulting in cell death and disease (Agarwal et al., 2005).

Superoxide Anion ($\cdot O_2^-$)

The superoxide radical ($\cdot O_2^-$) is a major ROS mostly produced from the mitochondrial electron transport chain (Valko et al., 2007). It is formed by the addition of a single electron to molecular oxygen (O_2) resulting in a very reactive unpaired electron state.

$$O_2 + e^- \longrightarrow \cdot O_2^-$$

This is the initial step in formation and propagation of ROS within and out of cells (Al-Gubory et al., in press). The $\cdot O_2^-$can react with other molecules and generate most of ROS. The most of its damage, superoxide anion achieves trough the production of ·OH (Shackelford et al., 2000).

$$2H_2O + \cdot O_2^- \longrightarrow \cdot OH + OH^- + O_2$$

Superoxide dismutase (SOD) detoxifies $\cdot O_2^-$ by its conversion to O_2 and H_2O_2 (Sorg, 2004).

$$2\cdot O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

Hydrogen Peroxide (H_2O_2)

$H_2O_2$is produced by intracellular reactions, in particular oxidative electron transport in the mitochondria (Shackelford et al., 2000). It plays a role in cellular reactions and intracellular signaling. Unlike superoxide anion, hydrogen peroxide is not a free radical but it is highly important much because it can cross biological membranes (Al-Gubory et al., in press).

H_2O_2 play a role as an intermediary in production of more reactive ROS molecules. Transition elements, such as iron (Fe) and copper (Cu) can interact with $\cdot O_2^-$ and $H_2O_2$to form even more reactive hydroxyl radical (·OH)in the metal ion-catalyzed Haber-Weis and Fenton reaction, respectively (Halliwell and Gutteridge, 1984; Sorg, 2004).

$$Fe^{3+}/Cu^{2+} + \cdot O_2^- \longrightarrow Fe^{2+} + O_2$$

$Fe^{2+}/Cu^{+} + H_2O_2 \longrightarrow {\cdot}OH + OH^{-} + Fe^{3+}$ (Fenton reaction)

The net reaction (Haber-Weis reaction):

Fe^{3+}/Cu^{2+}

$O_2^{-} + H_2O_2 \longrightarrow {\cdot}OH + OH^{-} + O_2$

H_2O_2 is removed by antioxidant enzymes, including catalases, glutathione peroxidases and peroxiredoxins (Nordberg and Arnér, 2001).

Hydroxyl Radical (·OH)

As present above in Fenton reaction, hydroxyl radical is formed from hydrogen peroxide in a reaction with metal ions(Fe^{2+} or Cu^{+}) which are usually bound to proteins, such as feritin, or other molecules. ·OHexpresses strong reactivity with biomolecules being able to damage biological system more then any other ROS (Betteridge, 2000). ·OH reacts rapidly with DNA and is known to activate certain oncogens (Shackelford et al., 2000).

Antioxidant System

Antioxidants are defined as any substance that prevents, delays or removes oxidative damage to a target molecule. They can be either synthesized *in vivo* or derived from a diet (Sordillo and Aitken, 2009). The antioxidant defense could be classified either as enzymatic and nonenzymatic antioxidants (Agarwal et al., 2005) or preventive and chain-breaking (Miller et al., 1993).

Preventive system includes metal-binding macromolecules and antioxidant enzymes. Metal-binding macromolecules such as transferin, ceruloplasmin and albumin remove metal catalysts of ROS reaction in extracellular fluid. Antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase act within cells and remove $O_2$and H_2O_2before they become available for Fenton reaction to produce more reactive ·OH radical (Halliwell, 1987). In spite of the action of preventive enzymes, some ROS may remain and cause deleterious effect on macromolecules. In this situation, there are some other antioxidant enzymes

that are chain-breaking and inhibit oxidative damage of macromolecules such as paraoxonase-1 (PON1), platelet-activating factor acetylhydrolase (PAF-AH) and lecitin: cholesterol acyltransferase (LCAT) that prevent and retard oxidative modification of lipoproteins (Parthasarathy et al., 1999; Link et al., 2007; Turk et al., 2004; 2005a,b; 2007; 2008a,b).

Chain-breaking antioxidants act after the initiation of chain reaction with ROS. They could be classified as lipid-soluble and water-soluble antioxidants including antioxidant vitamins, minerals, enzymes and other substances of endogenous or exogenous origin such as vitamin C, vitamin E, selenium, zinc, ubiquinone, taurine, hypotaurine, glutathione, beta carotene and carotene (Miller et al., 1993; Agarwal et al., 2005). Glutathione has been found to have great importance in bovine zygote and embryo development (de Matcos and Furnus, 2000). Aditionally, taurine has been found in human follicular and tubal fluid protecting the embryo from oxidative stress (Guerin et al., 2001).

ROS and Reproductive Processes

ROS and Steroidogenesis

Normal reproduction in cows depends on suitable concentrations of progesterone (P4) and estrogens at appropriate time (Miller et al., 1993). Steroidogenic enzymes depending on cytochrome P-450 are susceptible to peroxidation limiting synthesis of steroid hormones in steroidogenic tissues (Takayanagy et al., 1986). Mitochondrial cytochrome type enzymes are involved in the biosynthesis of steroidal compounds derived from cholesterol (Miller, 2005; Hanukoglu, 2006). These enzymes catalyze the reactions of cholesterol conversion to pregnenolone which is the first step in biosynthesis of steroid hormones (Hanukoglu, 2006). Androgens, estrogens and cortisol are all synthesized from pregnenolone by either of two metabolic pathways, which require several enzymes including 3β-hydroxysteroid dehydrogenase-isomerase, 17α-hydroxylase, 17,20-lyase, 21-hydroxilase, 11β-hydroxylase, 18α-hydroxylase and aromatase.17α-hydroxylase is required for both metabolic pathways (Miller et al., 1993). It has been found in *in vivo* study of human adrenal microsomes that 17α-hydroxylase and 17,20-lyase were five to six time more vulnerable to ROS than 3β-hydroxysteroid dehydrogenase-isomerase and 21-hydroxilase (Takayanagy et al., 1986). Therefore, ROS inactivation of these key enzymes could inhibit synthesis of androgen and

estrogens in reproductive tissues. Thus, suppression of androgens and estrogens induced by oxidative stress can impair reproduction in dairy cows.

ROS and the Ovary

Oxidative stress plays a role in the physiology of ovarian function. The oocyte exists in a follicular fluid which present a metabolically active environment comprising of granulosa cells, growth factors, steroid hormones, leukocytes and cytokines, all of which can produce ROS (Attaran et al., 2000). Several cells in the ovaries, such as endothelial cells, steroidogenic cells and phagocytic macrophages also produce ROS (Halliwell and Gutteridge, 1988). Limited knowledge is available of effect of ROS on oocyte maturation, fertilization and pregnancy (Das et al., 2006). ROS is supposed to be involved in metabolic processes in ovaries such as folliculogenesis, follicule maturation, ovulation and function of corpus luteum. Steroidogenic cells including theca cells, granulosa cells and hilus cells express particularly strong activity of oxidative enzymes (Agarwal, 2005). It seems that ROS might have different effects at different stages of embryo development (Pasqualotto and Pasqualotto, 2007). In human study on *in vitro*fertilization (IVF), Attaran et al. (2000) reported a beneficial role for ROS on conception cycles. Follicular fluid contains not only ROS but also antioxidants (Pasqualotto et al., 2004). SOD izoenzymes is an important scavenger of free radicals being in positive correlation with oocyte maturation (Tatemoto et al., 2004). Besides, in genetic manipulation studies in mice Ho et al. (1998) found that SOD-deficient mice had reduced fertility. Additionally, it has been found that decreased activity of glutathione peroxidase in follicular fluid may influence low ability of gametes to fertilize and low fertilization rates (Paszkowski et al., 2005). Concentration of ·NO in follicular fluid has been found to negatively associate with embryo quality and rate of cleavage (Bedaiwy et al., 2004). Higher ·NO concentration could be associated with implantation failure leading to lower pregnancy rates. Moreover, ·NO may induce apoptosis resulting in embryo fragmentation (Agarwal, 2005). Raised peroxidative processes in the follicle could have deleterious effect on oocyte maturation (Jozwik et al., 1999).

PATHOPHYSIOLOGY OF THE PERIPARTURIENT PERIOD

Pregnancy, parturition and the onset of lactation pose enormous physiological challenges to the homeostasisof dairy cows being a risk interval for their health and reproductive performance (Butler, 2000; Lucy, 2003). In late gestation, the dairy cow is in a largely anabolic metabolic state. Following calving, as lactation begins, the endocrine profile turns towards primarily catabolic metabolism (Taylor et al., 2003). Most of the metabolic diseases such as milk fever (clinical hypocalcaemia), ketosis, retained placenta, and displacement of abomasum occur within the first two week of lactation. In addition, some of infectious diseases, in particular mastitis, become clinically apparent during the first two weeks of lactation (Goff and Horst, 1997).

During the transition period from late pregnancy to early lactation, energy requirements that is needed for fetal growth and milk synthesis increase dramatically exceeding the amount of energy the cow can obtain from dietary sources. This dramatic increase in energy requirements makes dairy cows highly susceptible to negative energy balance (NEB) which commonly occurs in the transition period. The metabolic adaptation to NEB requires interactions of metabolic fuels and its failure may occur in various tissueslike the liver, adipose tissue and others (Herdt, 2000). Maternal metabolic accommodation for glucose and amino-acid requirements in late pregnancy and early lactation affects not only carbohydrate and protein metabolism but also lipid metabolism. Furthermore, the rates of hepatic gluconeogenesis and adipose fat mobilisation are greatly accelerated what is often associated with lipid metabolism disturbances (Bell, 1995). The state of NEB is further characterized by changes in blood metabolites, most noticeably increases in plasma non-esterified fatty acid (NEFA), betahydroxybutyrate (BHB) and urea (Grummer, 1993; Bell 1995; Rukkwamsuk et al. 1999; Pysera and Opalka, 2000). In addition, calcium and other minerals homeostasis are disrupted through parturition.Such disorders especially occur in cows which are over-conditioned at calving and exhibit decreased appetite, so they develop more severe NEB than cows of moderate conditioning (Rukkwamsuk et al. 1999).

These metabolic disorders in the periparturient period (3 weeks before calving to 3 weeks after calving) is associated with reproductive disorders in advanced lactation, such as increased interval to first ovulation after calving, decreased conception rate as well as a prolonged calving interval. The first ovulation after calving reflects recovery from the hormonal conditions in late pregnancy(Butler, 2000). From a number of investigations, during the first three weeks of lactation, NEB is highly correlated with the interval to first

ovulation (Butler, 2000). Thedegreeof NEB in the early postpartum period is related to endocrine disorders which causes a decrease in LH pulse frequency, decreased diameter of dominant follicles with low estradiol production and decreased systemic IGF-I and intra-follicular IGF-I availability. These disorders all together lead to increased interval to first estrus (Roche et al., 2000).NEB in the early postpartum period causes low glucose and insulin concentration.It is well known that insulin stimulate ovarian follicular cells development (Spicer et al., 1993; Simpson et al., 1994). Additionally, IGF-I has great impact to follicular development. The major amount of IGF-I in follicular fluid is derived from circulation (Perks et al., 1999) and its plasma concentration is directly related to energy status (Beam and Butler, 1999). Lower dominant follicles cause decreased secretion of estradiol leading to poor oocyte quality and weak estrus expression making the detection of estrus even more difficult. As a consequence, fertility is declined.

CYSTIC OVARIAN FOLLICLES

Definition

Cystic ovarian follicles (COF) in cows and heifers traditionally have been defined as an anovulatory fluid-filled hollow structures having diameter≥ 2.5 cm that persist for 10 or more days in the absence (Garverick, 1997; Tomašković et al., 2007) or in presence (Youngquist, 1994) of a corpus luteum which persists in one or both ovaries and accompanied by abnormal estrus behavior (irregular estrus intervals, nymphomania or anoestrus) (Ptaszynska, 2009). The absence of a corpus luteum is requirement, which is not always fulfilled (Al-Dahash and David, 1977). Non-steroidogenic cysts which are hormonally inactive do not influence the regular estrus cycle, so they can occur together with a corpus luteum.

There is still no consensus about cyst diameter, although many authors use a diameter of 2.0 cm as a minimum (Boryczko et al., 1995; Fleischer et al., 2001). However it has been clear though that this definition needs to be revised. Recent data using ultrasonography indicate that typically follicles ovulate at average size of 1.6 to 1.9 cm in dairy cows (Bleach et al., 2004; Lopez at al., 2004; Garverick, 2007). So follicles that persist at that diameter or greater may be consider cystic (Vanholder et al., 2006) which is the reason for the term cystic ovarian follicles to be common use, rather than ovarian cysts(Ptaszynska, 2009) or cystic ovarian disease (Parkinson, 2009). Finally,

COF may be defined as follicles with diameter of at least 2.0 cm that are present on one or both ovaries in the absence of any luteal tissue and that clearly interfere with normal ovarian cyclicity (Vanholder et al., 2006).

Many researchers showed that COF are actually dynamic structures which can regress and be replaced by new cysts (Hamilton et al., 1995; Yoshioka et al., 1996). Factors which affect the cysts regression are still unknown (Peter, 2004), although changes in mean LH concentration seem to be involved (Hamilton et al., 1995). It seems that an abnormality in FSH secretion is not involved in this disorder (Hamilton et al., 1995).

Sakaguchi et al (2006) suggested that the occurrence of cysts symptoms is rather a consequence of generalized disturbance of ovarian function ranging from anovulatory follicle waves with normal duration, through temporary appearance of anovulatory cystic structures which develop and regress in a clinical manner, to anovulatory cysts that persist in the ovary for extended period of time. Hence, many cows develop large fluid-filled structures in the ovaries during the immediate postpartum period. These normally regress spontaneously but present as a clinical problem when they cause aberrant behavior or alterations of the estrus cycle.

COF are considered to be one of the most frequent and important causes of ovarian disorders and reproductive failure in modern high yielding dairy cows (Gossen and Hoedemaker, 2006; Vanholder et al., 2006). There is a severe economic loss to dairy industry due to COF resulting in reduced milk production, veterinary expenses, increased interval between calving and conception, increased involuntary culling rates (Gröhn et al., 1997; Moss et al., 2002; Silvia et al., 2002). Most of COF that develop during the early postpartum period regress spontaneously. However, it is difficult to decide when it would be more cost-effect to treat COF than to wait for spontaneous recovery (Lopez-Gatius et al., 2002). Despite a higher incidence of spontaneous recovery, the increased interval between calving and conception due to COF still remains a problem in dairy industry (Gröhn et al., 1997).

Incidence, Etiology and Pathogenesis

COF are the most common reproductive disorder in dairy cows with incidence of approximately 6-30% being significantly higher in the early postpartum period (Laporte et al., 1994; Garverick, 1997; Opsomer et al., 1998). However, 60% of cystic cows recover spontaneously prior to the first postpartum ovulation (Ijaz et al., 1987). Despite this high self-recovery rate, an

importance of COF in dairy cows is considerable (Lopes-Gatius et al., 2002). Furthermore, the incidence of COF depends on parity. In the studies by Hacket and Batra (1985)(n=1830) and Fleischer et al., (2001) (n=2197) the incidence of COF in the lactation period was 5.7% and 7.4% in heifers, respectively, and 18% and 13.7% in multiparous cows, respectively.

Early resumption of ovarian cyclicity is beneficial for fertility. By delaying ovarian cyclicity COF increase period to first insemination, days open and inter-calving period (Shrestha et al., 2004; Tomašković et al., 2007). In addition, COF decrease the pregnancy rate after first insemination and increase the number of services per conception(Shrestha et al., 2004).

COF are important causes of subfertility and infertility in cows and heifers. Since there is no single cause of COF, an interaction between hereditary predisposition, milk yield, age, season, nutrition management, stress and negative energy balanceis important in COF development.All mentioned factors are considered to be predisposing factors (Parkinson, 2009; Ptaszynska, 2009).

A genetic predispositionfor COF exists but the heritability is rather low being between 0.07 and 0.12 (Cole et al., 1986; Zwald, 2004). In order to reduce the incidence of COF,genetic selection can be useful, despite the low heritability (Vanholder et al., 2006). The incidence of COF has been dramatically reduced within a breed by avoiding the use of cows that have had COF as bull mothers and by avoiding the use of bulls that have bred daughters that have the disease as bull sires (Bane, 1964). COF are more common in Holstein-Friesians than in other dairy breeds and they are rare in beef breeds (Parkinson, 2009).

Many studies found higher incidence of COF in some herds and it is more common in winter than in other seasons of the year. This may reflect the fact that the majority of cows calve in the autumn and thus reach the peak yield in winter. It may reflect a lack of exercise, excess dietary protein and/or the effect of photoperiod (Peter, 2004).

Postpartum diseases such as ketosis, dystocia, placental retention, stress, twin births, milk fever, thyroid glanddisorder due to iodideinsufficient food intakeand postpartum uterine infections have been associated with increased risk factors for COF (Roberts, 1986; Laporte et al, 1994, Ptaszynska, 2009). Cows who had been suffering from endometritis developed abnormal patterns of ovarian activity (Mateus et al., 2002). In accordance with these results Gossen and Hoedemaker (2006) found out that cows with endometritis developed significantly more often COF that cows without endometritis.In endometritis,endotoxins or increased cortisol level induced by endotoxins can

cause insufficient release of LH surge and cause COF development (Tomašković et al., 2007).Furthermore, LH surge mechanism can be attenuated by stress and by activation the hypothalamic-pituitary-adrenal corticoid axis (Nanda et al., 1990).

The incidence of COF is the greatest in cows aged between 4 and 6 years (Roberts, 1986) during the first months postpartum that is in correlation with stress caused by high milk production influencing LH release failure (Lucy, 2001). High milk production was suggested to be positively correlated with the development of COF. Cows with lower milk production which suffer from COF during postpartum period recover spontaneously more often then cows with higher milk production. (Lopez-Gatius et al., 2002). Therefore, high milk production seems to be a risk factor for COF formation. The time of COF formation seems to have an influence on reproductive performance in dairy cows. COF diagnosed after the puerperium had a negative effect on fertility whereas COF diagnosed during the postpartum did not affect reproduction (Gossen and Hoedemaker, 2006).

Some data indicate the existence of a genetic background to COF. Since a genetic correlation between COF and milk production has been established, selection of cows for milk production will increase the incidence of COF (Hooijer et al., 2001).

Nutritional factors that influence COF development include β-carotene deficiency. Besides, phyto-estrogens and potassium sufficiency play a role as well (Tomašković et al., 2007; Ptaszynska, 2009). Although it has been suggested that β-carotene may reduce the incidence of COF (Lotthammer, 1979), that was not supported by other authors (Marcek et al., 1985) who found no benefit from such supplementation in cows' diets. Excessive dietary protein may also have a direct effect on the incidence of COF (Ashmawy et al., 1992). Insufficient food intake has a direct effect on follicle growth and development. Dietary effect on ovarian function manifested on: hypothalamus (regulate synthesis and releasing of GnRH), pituitary (regulate synthesis and releasing of FSH, LH and growth hormone (GH)) and ovaries (regulate follicle growth and synthesis of steroid hormones) (Diskin et al., 2003).

Nutritional deficiencies (negative energy balance, NEB) are thought to be one of the most important factors contributing to the formation of COF during the early postpartum period (Vanholder et al., 2006). At this time, energy requirements to sustain milk yield are higher than energy intake thus causing a NEB. NEB is accompanied by several hormonal and metabolic adaptations affecting ovarian function (Beam and Butler, 1999). Some cows can compensate for higher milk production through greater dry matter intake

reducing the influence of milk yield on energy balance (Lucy, 2001). That could explain why some other researches (Bartlett et al 1986; Nanda et al., 1989)have not observed a correlation between ovarian cysts and milk yield. Moreover, it seems that a correlation between COF incidence and a magnitude and/or duration of NEB occurs (Vanholder et al., 2006). During the NEB, peripheral plasma IGF-I, insulin, glucose and leptin concentrations are reduced, while concentrations of metabolites such as non-esterified fatty acids (NEFA) (Rukkwamsuk et al., 2000) and β-hydroxybutyrate (BHB) are increased (Leroy et al., 2004). NEB is a metabolic disorderthat affects high yielding dairy cows, it undermines health and it has prolonged effects (few months) on fertility. NEB duration and its intensity vary depend on genetic predisposition, body condition prior to calving, milk production and foodintake(Grummer et al., 1995).NEB starts priorto calvingand reaches the highest peak during the first month of lactation(Prandi et al., 1999). It is considered that during the first 3-4 weeks post partum, NEB is in high correlation withthe time of the first post partum ovulation (Butler, 2000). Although elevated serum ketone concentrations increased the risk for the formation of cystic follicles in post partum dairy cows, they were not found to exert any negative effects on bovine follicle cells in vitro (Vanholder et al., 2006b). Therefore, ketone concentrations in the postpartum dairy cow seem to be indicator of the NEB severity, rather than a mediator of the negative effects of the NEB on reproduction at the ovarian level (Ptaszynska, 2009).

The IGF-system plays an important role in follicle growth and development (Spicer and Echternkamp, 1995). Diet is the main factor of the regulation of insulin like growth factor I (IGF-I) production in liver, that has an important role as metabolic signal in the first postpartum ovulation regulation (Braw-Tal et al., 2004).

The correlation between low IGF-I plasma leveland lower conception rate supports the thesis that IGF system has an important role in a genital tract (Wathes et al., 1998). The cows that have low IGF-I and insulin level in early puerperium will probably have ovarian cyclicity disorders and higher prevalence of COF development and consequently lower conception rate (Pushpakumara et al., 2003; Vanholder et al., 2006).

Leptin is a hormoneproduced by adipocytes and is regarded as the ultimate factor linking metabolic status and reproduction (Barash et al., 1996). Depending on the metabolic state of the animal, it either has a stimulatory effect or none on hypothalamic-pituitary function in cows (Amstalden et al., 2005). Minimum permissive concentration of leptin seems to be required to induce the first postpartum LH surge (Block et al., 2001). Therefore, leptin

may play an important role in early puerperium COF development (Vanholder et al., 2006).

COF etiology is very complex. This dysfunction has a multifactorial etiology, in which genetic, phenotypic and environmental factors are involved (Peter, 2004; Tomašković et al., 2007). COF may result from function disorders at both ovary/follicle and hypothalamus/pituitary levels. It is generally accepted that the cause of COF is a dysfunction of the reproductive neuro-endocrine system, resulting in the aberrant patterns of LH secretion during the development of a dominant follicle and a failure of LH surge mechanism (Peter, 2004). It could be either absent, insufficient in magnitude, or occurs at the inappropriate time during the maturation of the dominant follicle (Ptaszynska, 2009).It is believed that the preovulatory LH surge is absent or attenuated (Hamilton et al., 1995), as a result of partial or complete failure of estrogens to elicit a normal positive feedback secretion of LH (Nanda et al., 1991). This appears to be due to failure or lack of sensitivity of the hypothalamic surge-generating centre to estrogens (Garverick, 2007) and a failure of GnRH release rather than a lack of pituitary LH content or a lack of pituitaryresponsiveness to LH (Vanholder et al., 2005). The primary cause could be in the hypothalamus, which fails to release a surge of GnRH in response to an estrogens stimulus. Hypothalamic insensitivity to estrogens may be induced by intermediate (subluteal) concentrations of circulating progesterone. If progesterone is administered at intermediate levels (0.5-2 ng/mL), it will block the LH surge, prevent ovulation, and result in the formation of a follicle with a greater diameter and persistency than in physiological condition. (Hatler et al., 2003).

Primary dysfunction at the level of the follicle may disrupt the hypothalamic-pituitary-ovarian axis causing the formation of COF. Alterations in LH-receptor expression and content may cause anovulation of the follicle. Besides this, alteration in steroidogenesis by the dominant follicle may also be involved in cystic degeneration.

The major factor in the development of COF is alterations in the biochemical activity of the follicle (Parkinson, 2009). There some evidenceof a reduced population of LH receptors in the granulosa of cysts in comparison to normal follicles (Kawate et al., 2004). There is also evidence for reduced estrogen receptors in the granulosa of cysts (Salvetti, 2007).

If discussing the pathogenesisof COF, a distinction may be made between a primary defect in the hypothalamus-pituitary and a primary defect at the level of the ovary in the follicle itself (Vanholder et al., 2006). An aberrant LH surge is likely to be the trigger for the development of COF. Abnormal LH

release seems to be caused by an altered feedback mechanism of estrogens on the hypothalamus-pituitary. The malfunctioning of the feedback mechanism can be caused by factors directly interfering at the hypothalamic-pituitary level or by an altered follicle growth and development disrupting the hypothalamic-pituitary-ovarian axis (Vanholder et al., 2006).

COF Classification and Clinical Signs

COF are traditionally classified as either follicular or luteal cysts but these are considered to be different forms of the same disorder and this classification is based on their stadium of luteinisation (Cook et al., 1990; Parkinson, 2009). COF could be single or multiple. Follicular cysts can occur more often, in approximately 70% of cases, while luteal cysts occur in only 30% of cases.

Previously, it was considered that cysts of corpus luteum occurred, but now it is proved that center of the corpus luteum could be occupied by cavity. That condition is normal and could be seen even in 25% cows and heifers (Noakes, 1998).

Clinical signs of COF vary and depend on the extent of luteinisation of the cyst. In most cases (62-85%), cows with COF are anoestrus, especially during the postpartum period (Watson and Cliff, 1997; Ptaszynska, 2009) as a result of the production of progesterone by luteinized cysts. Nymphomania and irregular cycles with relaxation of the broad pelvic ligaments and development of masculine physical traits are other signs of the presence of estrogenically active follicular cyst are also common, especially later in lactation (Kasari et al., 1996; Ptaszynska, 2009). Also, it is possible combination of anoestrus, nymphomania and irregular cycles (Tomašković et al., 2007).

Follicular cysts follow anovulation of mature (de Grafiaan follicle), and instead ovulation follicle continue to grow and often release estrogens and androgens (Tomašković et al., 2007). Follicular cysts are thin-walled (less then 3 mm), soft or tense fluid-filled fluctuating structures, ≥2.5 cm diameter that persist (Figure 1). Follicular cysts may be single or, commonly multiple and associated with low peripheral progesterone concentrations in blood or milk. Affected cows are either anoestrus or nymphomaniac. Also, it is possible a combination of anoestrus and irregular oestrus cycles (Parkinson, 2009). Sometimes, affected cows and heifers can exhibit regular estrus cycles, but it is very rare (Tomašković et al., 2007).

Nymphomaniac cows showing attempt to ride other cows and, as with cows in estrus, will stand to be mounted by other cows (Noakes, 1998).

Affected cows have excessive, prolonged signs of estrus which sometimes last for several days. These cows sometimes calm down after two or three days in order to express estrus again in more or less regular intervals. Spontaneous recovery of affected cows occurs frequently in the early postpartum period and cows can have conception without any treatment (Parkinson, 2009). If cysts persist for a longer period, clinical signs could often be complicated with catarrhal or purulent endometritisthat develops as a consequence of weaker immune defenseability of affected cows, endometrium degeneration and secondary bacterial infection. Following rectal palpation on one or both ovaries, it could be found soft or tense thin-walled fluid-filled fluctuating structures ≥ 2,5 cm (from cherry size until size of hen egg) (Tomašković et al., 2009).



Figure 1. Ultrasound image of follicular cyst (Tomašković et al., 2007).

Follicular cysts are thin-walled and during rectal palpationcan easily burst.Following vaginal examination, findings are similar to those in estrus: edematous swelling of the vulva, cervix is usually bigger, more edematous than in normal estrus, central cervix crease (*portio vaginalis cervicis)* is hyperemic with frequent and copious clear mucus or muco-purulent vaginal discharge and a shortened interval between successive heats. The uterus is big, soft, slack and atonic. Because of their excessive sexual activity they have a generally disruptive effect upon the rest of the herd, making accurate estrus detection difficult.

If estrus last for several weeks, affected cows changing their behavior, become more aggressive, disturbing other animals, attack them, and sometimes they can attack objects in their environment. Affected cows have

nervous disposition with depressed milk yield and loss of body condition. Furthermore, owing to the relaxation of the pelvic ligaments, they are prone to pelvic and hip fractures (Noakes, 1998).

In the last 20 to 35 years it has been changing in behavioral patterns. Dobson et al. (1977) and Booth, (1988) were found that out of total number of affected cows with follicle cysts only approximately 27% were nymphomaniac, while 73% cows were acyclic.

It is known that luteal cystsdevelop in the presence of LH concentrations that are insufficient to induce ovulation, but capable of causing luteinisation of the follicular walls. Luteal cyst like follicular cyst develops from mature (de Grafiaan follicle) and instead ovulation follicle continues to grow and later become luteinized. Luteal cysts are thick-walled fluid-filled structures $\geq$ 2.5 cm diameter that persist. Thickness of cyst wall is more than 3 mm and they are usually single (Figure 2). The consequence of luteal cyst is high peripheral progesterone concentration in blood or milk (Parkinson, 2009). The structure functions as a persistent corpus luteum (Noakes, 1998). All affected cows are almost invariably associated with anoestrus (Tomašković et al., 2007; Ptaszynska, 2009). It is difficult to understand why it does not regress under the influence of endogenous $PGF_{2\alpha}$, since it will regress under the influence of exogenous $PGF_{2\alpha}$ (Noakes, 1998). If affected cows are left untreated then a proportion of them will become virilized (Parkinson, 2009) and moo like bulls (Tomašković et al., 2007). These individuals will develop a masculine conformation and will attempt to mount other cows, but unlike the nymphomaniac cow they will not stand to be mounted by other cows (Noakes, 1998).

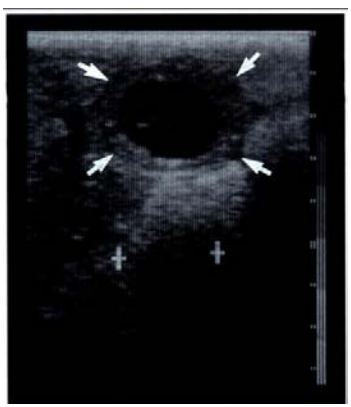


Figure 2. Ultrasound image of luteal cyst (Tomašković et al., 2007).

Distribution and Diagnosis

COF may be presented in one or both ovaries. More cysts are identified in the right ovary then in the left ovary (Parkinson, 2009). Al-Dahash and David (1977) found in a survey of over 8000 genital tracts, that over 53% out of the 307 cows suffered from COF had a single cyst and over 46% had multiple cysts. The majority of COF were between 2.5 and 3 cm in diameter.

Rectal palpation, ultrasonography and determination of progesterone concentrations in plasma or milk are common diagnostic tools for COF (Hooijer, 2003).For a long time, rectal palpation was the only way to diagnose COF. By rectal palpation identification and differentiation of COF is not very accurate because there are numerous of other fluid-filled structures that may be present on the ovary and need to be differentiated from COF. However, luteal cysts have a ticker wall which only few experienced clinicians are able to detect by rectal palpation (Ptaszynska, 2009). In opposite to rectal palpation, ultrasonography is generally more accurate diagnostic method (Douthwaite and Dobson, 2000). Real time B-mode ultrasonography was introduced to veterinary practice in the early 1980s and offered a new tool for early pregnancy diagnosis (Taverne, 1984). Nowadays, it is used for different species not only to detect pregnancy, but also to assess pathological or physiological structures of the genital tract (Kähn, 1991). Care should be taken to not to confuse luteal cysts with hollow corpus luteum, which are not pathological at all (Ptaszynska, 2009). The assessment of cow ovarian structures by ultrasonography has been developingin the last 20 years (Kähn, 1991; Hanzen et al., 2000; Dobranić et al., 2008).

The type of COF can be confirmed by measuring peripheral progesterone concentration by RIA or ELISA methods in blood or milk (>2 ng/mL in milk (Booth, 1988)or >1.0 ng/mL in plasma/serum (Caroll et al., 1990; Farin et al., 1992) are considered to be indicative for a luteal cyst). Opsomer et al. (1999) used milk progesterone analysis for examination postpartal ovarian cyclicity and stated that several so-called cystic structures are normally functional corpus luteum. The accuracy of manual palpation or ultrasonography can be increased by obtaining information about the reproductive history of the cow and by palpation of the uterine horns, vaginal examination, or progesterone determination (Hanzen et al., 2000; Tomašković et al., 2007). Progesterone determination has confirmed abattoir data that follicular cysts are two or three times more common than luteal cysts (Booth, 1988).

Prevention

By careful genetic selection, improvements have been made by eliminating bulls that have sired daughters subsequently suffering from COF. Ideally, cows should not be treated for COF and their progeny should not be used for breeding. Unfortunately, this makes confusion in dairy herd management because affected cows are usually the best milk producers (Parkinson, 2009). Prevention of COF can be approached by identifying and eliminating the contributory causes of the disorder (peripaturient stress, nutritional inadequacies and uterine infections). Prophylactic use of GnRH has shown some success in reducing the prevalence of COF in herds (Zaied et al., 1980). Kesler and Garverick, (1982) recommended that all cows should be treated with 100-200 μg of GnRH 12-14 days after calving but cost-effective has not been calculated (Britt et al., 1977). Earlier application is ineffective because the pituitary gland is not capable to release LH in response to GnRH before 12-14 days after calving (Ptaszynska, 2009).

Prognosis and Treatment

COF treatment is the most successful in young animals, in early postpartum period and in cases of single and one side COF. By using the most recent treatment methods it is possible to cure numerous affected cows and heifers.

In spite of a relatively high self-recovery rate, untreated COF, even diagnosed, can extend the calving to conception interval for 64 days, leading to economic losses of $55 to $160 per lactation (Bartolome et al., 2005). Every COF treatment startswithremoval of detected causes, which means diet corrections, pasture, selenium andvitamins A and E application (1 to 2 million I.U. vitamin A and 200 mg vitamin E). In case of mineral deficit in cow's diet, supplementation by minerals can be applied (Tomašković et al., 2007).

The earliest method of treating cysts was manual rupture at rectal palpation (Parkinson, 2009). Although rupture sometimes occurs inadvertently it should not be done intentionally because it is not possible to remove the cause of COF formation and COF will develop again (Tomašković et al., 2007). Manual rupture is only physical elimination of the cystic follicle without the stimulation of normal follicular turnover and the induction of ovulation from a new follicular wave that produces the desired result. For this reason, manual rupture of the COF often fails to restore cyclicity (Ptaszynska,

2009). Besides, that procedure can cause trauma or hemorrhage which might result in ovarobursal adhesions (Parkinson, 2009). If during rectal palpation COF ruptures accidentally, it should be waiting for a new estrus cycle. Cow can be inseminated with GnRH administration at the same time in order to induce ovulation (Tomašković et al., 2007).

The choice of the treatment and success will depend on some extent upon the cyst type:

Follicular Cyst

1. GnRH application is the first treatment of choice (Tomašković et al., 2007). It acts by stimulating the pituitary gland to release LH and FSH (Ptaszynska, 2009). A maximal plasma LH concentration is reached within 90 to 150 minutes after application (Kruip et al., 1977). The induced LH surge leads to luteinization of the COF (Jeffocate and Ayliffe, 1995). However, the use of higher dose of GnRH (0.5-1.0 mg) may induced some COF to ovulate with a consequence of corpus luteum formation (Berchtold et al., 1980). Whatever occurs, the result is an increase in progesterone concentration, which causes negative feedback on LH secretion and resets the sensitivity of the pituitary to estrogens (Gümen and Wilbank., 2002). Moreover, the GnRH-induced increase in FSH concentration causes recruitment of a follicular wave that usually restores normal cyclicity. Following treatment, 65 do 90% of cows will come into estrus between 3-15 days (Nanda et al., 1989) and 18-23 days after application of GnRH (Ijaz et al., 1987; Ptaszynska, 2009). COF recur in 8% (Nanda et al., 1988) to 16% (Watson, 1998) of cows. GnRH has become available during last 15 years, initially as a synthetic decapeptide that has an identical molecular structure to the naturally occurring hormone but also as a nonapeptide analogue with a longer biological half-life(Noakes, 1998). The used dosage of GnRH varies between 50 to 500 μg depending on the manufacture's recommendation (Hooijer, 2003). Seguin et al. (1976) demonstrated that the serum progesterone levels increase by more than 2 ng/mL in the 11^{th} day post treatment with 50, 100, 150 and 250 μg GnRH. Once luteinization of the COF has occurred initiated by the GnRH, luteal tissue is developed within 9 days post treatment. The resulting corpus luteum should then respond to the subsequent $PGF_{2\alpha}$ treatment, and a new estrus cycles begins (Garverick, 2007; Ptaszynska, 2009).

2. Intravenous hCG application is another treatment possibility. hCG is a gonadotrophin with strong LH activity. It has a half-life in cows nearly 48 hours, and thus exerts a long-acting luteotropic effect directly on the cyst, and it is frequently reserved for recurrent cases (Ptaszynska, 2009). Like GnRH, hCG will cause COF luteinization and sensitivity to $PGF_{2\alpha}$ (Tomašković et al., 2007). Following treatment, 75% of cows will come into estrus within 3 weeks (Dobson et al., 1977). GnRH and hCG elicit equivalent endocrine and clinical responses, but GnRH has an advantage over hCG in its minimal antigenicity (Drost and Thatcher, 1992). Thereby, frequently COF therapy by hCG will cause antibody development on hCG hormone and many cows will not respond any more (Tomašković et al., 2007). Cows that cannot come into estrus within 23 days, after GnRH or hCG treatment have to be examined and treated again, if necessary. The same could be applied to cows which show signs of estrus within 14 days, since this indicates that they failed to respond to the first injection (Ptaszynska, 2009).
3. A classic Ovsynch protocol can be used for the treatment of COF in lactating dairy cows. Synchronization of ovulation and timed insemination with an Ovsynch protocol resulted in pregnancy rates similar to those of estrus within 7 days (Bartolome et al., 2000). Further studies by De Vries et al., (2006) and De Rensis et al., (2008) confirmed the suitability of Ovsynch-type protocols for the treatment of COF in dairy cows.
4. Alternatively, follicular cysts can be treated with application of progestagens. It is especially effective in nymphomaniacal cows. Progestagens could be in form of ear-implant, intra-vaginal device (Hooijer, 2003; Ambrose et al., 2004) or injections with prolonged activity in oil suspension (Tomašković et al., 2007). Progestagens induce atresia of COF by suppressing FSH and LH support via steroid negative feedback mechanism (Hooijer, 2003). Signs of nymphomania abate within 24 hours and cysts gradually regress. Thereupon, signs of estrus with ovulation and corpus luteum formation occur in the next 10-12 days (Parkinson, 2009). Cows come into estrus in 2 to 5 days after withdrawal of progestagens. Due to progestagen withdrawal, it is possible to administrate GnRH for inducing ovulation and corpus luteum formation (Tomašković et al., 2007). Progesterone absorbed from the any application form suppresses the gonadotrophin support that is required for maintenance

of the COF, resulting in its demise (Noakes, 1998). Furthermore, increased peripheral progesterone concentrations result in a lowering pulsate LH secretion and a restoration of the ability of the hypothalamus-pituitary axis to generate an LH surge in response to estrogens (Todoroki and Kaneko, 2006).

5. hCG or GnRH in combination with progestagens can also be used in COF treatment(Kupfer el al., 1991; Ambrose et al., 2004). Several studies have indicated. Various studies have indicated that exposure of the effector cells of the ovarian follicle to sufficient levels of progesterone is essential for their sensitization to further gonadotrophin stimulation, further luteinization of cyst and sensitivity on $PGF_{2\alpha}$. Therefore the use of progestagens in combination with GnRH or hCG is logical treatment for follicular cysts and has led to very encouraging results (Ambrose et al., 2004; Tomašković et al., 2007).

Luteal Cysts

Luteal cysts can be treated like follicular cysts with GnRH, hCG and progestagens, but in comparison to follicular cysts luteal cyst can be treated with $PGF_{2\alpha}$. Results after GnRH of hCG application are fairly successful and approximately equal those like follicular cysts treatment. However, results with progestagens have been variable and generally progestagens by itself is relatively ineffective in treating luteal cysts (Todoroki et al., 2001).

The most logical way to treat luteal cysts is the use of $PGF_{2\alpha}$, although there is still no explanation for the failure of cows to respond to their endogenous $PGF_{2\alpha}$(Noakes, 1998). In 96% of treated cows, Dobson et al. (1977) found regression of cyst; the majorityof cows came into estrus within 3 to 5 days and 56% of the cows conceived, at a mean treatment-to-conception interval of 27 days. Tomašković et al. (2007) claimed that the effective of that method is between 60 to 90% of affected cows. The response and cure rate of this treatment depend on the presence of luteal tissue and diagnostic accuracy (Ptaszynska, 2009).

POSTPARTURIENT UTERINE DISEASES

Post parturient inflammation of the uterus, described as *acute* or *chronic metritis* aretwo the most common postpartum disorders in dairy cattle. The other two are *subclinical metritis* and *pyometra*.

Endometritis is inflammation of the muscular and endometrial layers of the uterus most commonly observed during the estrus and metestrus. Pathologic uterine infections that persist into the intermediate postpartum period are referred as endometritis (Scrollavezza et al., 1997). Endometritis can also results from pyometra or by introduction of pathogens during artificial or natural insemination (Scrollavezza et al., 1997).

Like the most researchers and clinicians all over the world, we have to accept the infection and to some extent the inflammation of the uterus during and after parturition as a physiological process (Lewis, 1997). Probably more than 90% cows have some kind of uterine infection early post partum. Good news are that more than 70% of those cases will be self-cured as soon as cows will boost their immune system and start normal lactation with no signs of negative energy balance (NEB).

Metritis

Metritis in dairy cows is an important disease, because it can increase the calving-to-conception interval (Bartlett et al., 1986; Erb et al., 1981; Fishwick, 1997), decrease milk yield (Coleman et al., 1985), increase culling (Bartlett et al., 1986; Coleman et al., 1985; Dohoo and Martin, 1984; Dohoo et al., 1983; Dohoo et al., 1984), increase veterinary service and drug use costs (Drillich et al., 2001; Drillich et al., 2005; Drillich et al., 2005a; Drillich et al., 2006). The occurrence of metritis and its economic impact might be decreased by manipulating risk factors for it.

Etiology of metritis is multicausal but the most important aerobic microorganism involved in metritis is *Actynomices pyogenes* often in conjunction with gram negative anaerobes *Fusobacterium necroforum and Bacterioides spp*. Infections with *Bacillus spp., Pasteurella spp., Pseudomonas spp.*, staphylococci and streptococci cause acute metritis but are the predominant factor in the onset of the chronic metritis and endometritis(Scrollavezza et al., 1997).

Table 1. Results of a multivariable analysis of risk factors for metritis on data, from 102,060 Danish dairy cows, collected during 1993–1994 (from Bruun et al., 2002).

Risk factor	Level	β	S.E.	P[a]	Odds ratio[b]	Conf. interval
	1	-0.017	0.11	<0.001	1.0b	0.8-1.2
	2	-0.506	0.12		0.6c	0.5-0.8
	3	-0.058	0.12		0.9b	0.7-1.2
Parity	≥4	0.000	0.00		1.0b	
	No	0.478	0.13	<0.001	1.6b	1.3-2.1
	Dry cows	0.762	0.24		2.1b	1.4-3.4
	Exercise area	0.627	0.34		1.9bc	1.0-3.6
Grazing	Yes	0.000	0.00			
Calving season	Nov-April	0.204	0.08	0.010	1.2b	1.0-1.4
	May-October	0.000			1.0c	
	Yes	1.101	0.15	<0.001	3.0b	2.3-4.0
Dystocia	No	0.000			1.0c	
	Yes	0.903	0.18	<0.001	2.5b	1.8-3.5
Repro. disease	No	0.000			1.0c	
	Yes	-0.755	0.51	0.013	0.5b	0.2-1.3
Ketosis	No	0.000			1.0c	
	Large, Yes	2.288	0.18	0.05	9.9b	6.9-14.1
	Jersey, Yes	2.380	0.27		10.8b	6.4-18.3
	Large, No	1.047	0.16		2.9c	2.1-3.9
Breed X Ret. Placenta	Jersey, No	0.000	0.00		1.0d	

Various risk factors for metritis have been identified: some are controversial, while others are consistent in the literature. The two of them which consistently have been identified are dystocia and retained placenta (Coleman et al., 1985; Curtis et al., 1985; Dohoo et al., 1983; Kaneene and Miller, 1995). Other suggested risk factors are: herd size (Kaneene and Miller, 1995;Roine and Saloniemi, 1978), age(Dohoo et al., 1983; Erb and Martin, 1980a; Erb and Martin, 1980b; Etherington et al., 1985), parity(Grohn et al., 1986; Markusfeld, 1984;Markusfeld 1987;Rajala and Grohn, 1998;Saloniemi et al., 1986), ketosis (Curtis et al., 1985; Kaneene and Miller, 1995; Markusfeld 1987), milk fever(Roine and Saloniemi, 1978;Saloniemi et al.,

1986), housing(Coleman et al., 1985) and calving season (Erb et al., 1981; Etherington et al., 1985; Grohn et al., 1986; Markusfeld, 1984; Markusfeld 1987;Martinez and Thibier, 1984). However, the herd-management factors grazing, farmer supervision at night for calving cows, and farmer intervention at calving and their influence on metritis have not been investigated previously. Table 1 shows results from study conducted during 1993-1994 by Bruun et al. (2002).

The risk factors significantly associated with metritis were: parity, grazing, calving season, dystocia, reproductive disease, ketosis, and the interaction between retained placenta and breed (Bruun et al., 2002). The significance of risk factors was evaluated using a score test which is a good approximation of the log likelihood-ratio test. However, the confidence limits were generated using Wald's approximation. Therefore, a risk factor could be significant in the score test, even though the Wald's confidence limits include 1.0 (as is the case for ketosis).

Risk Factors for Metritis

Reproductive Diseases

Dystocia and reproductive diseases both increased the odds of metritis. Dystocia can increase the risk of trauma to the uterine wall and thereby increase the odds of metritis. Also, calving assistance can increase the risk of introducing infection. Reproductive disease could increase the risk of infection (and thereby the odds of metritis), so this finding was not surprising and is supported by the literature (Coleman et al., 1985; Curtis et al., 1985; Erb et al., 1981; Etherington et al., 1985; Kaneene and Miller 1995).

Metabolic Disorders

The most common metabolic disorders are ketosis, milk fever and left displaced abomasum (LDA). All of these metabolic disorders will lead to stress and decreased immunological status of the cows. Regarding that facts they may become a risk factors for postpartum uterine infection and lead to postpartum metritis (Bačić et al., 2006).

Unexpectedly, ketosis seemed to be protective for metritis in study of Bruun et al. (2002). This might arise from inaccurate reporting dates of

disease; also, for cows with both diseases on the same day, it could be that only ketosis gets reported because it is probably considered the more important of the two. In the literature, some studies found ketosis to be a risk factor for metritis (Kaneene and Miller 1995;Markusfeld, 1987), whereas others have found no association (Curtis et al., 1985;Markusfeld, 1984) between metritis and ketosis in their cow-based model and a weak association (P=0.06) in the herd-based model. However, in their study, the specific dates for metritis were not recorded (the recording used was whether a cow had metritis within a month or not). In one study with positive association between ketosis and metritis, the measure for ketosis was ketonuria which means that subclinical cases of ketosis were included (Markusfeld, 1987). This could change the relationship to metritis. There was no association between milk fever and metritis, which is supported in the literature (Kaneene and Miller 1995). However, a positive association between metritis and milk fever also has been seen (Saloniemi et al., 1986). The time ordering of the disease variables was probably good in the study by Markusfeld, (1987), because cows were monitored daily by veterinarians and no treatment was performed by farmers; therefore these results could be the most reliable.

Retained Placenta and Breed Interaction

Retained placenta (RP) is a failure of the fetal membranes to be expelled within 12 to 24 hours after parturition. Metritis an inflammation or infection of the uterus is often associated with RP.

The effect of retained placenta has in the previous studies been shown to largely increase the odds of metritis (Coleman et al., 1985; Curtis et al., 1985; Erb et al., 1981; Etherington et al., 1985; Kaneene and Miller 1995;Saloniemi et al., 1986). However, because most studies include mainly one breed of cows like Holstein Friesian (Curtis et al., 1985; Erb et al., 1981) and Finnish Ayrshire (Grohn et al., 1986;Rajala and Grohn, 1998**;** Saloniemi et al., 1986), the retained placenta–breed interaction has not been tested. Cows with retained placenta had higher odds of metritis irrespective of breed. Also RP was associated indirectly with greater occurrence of cystic ovaries, lower milk yield and greater culling; all were mediated through metritis (Erb et al., 1981). Multiple physiological and nutritional factors have been associated with retained placenta and metritis. Dystocia in heifers increased the risk of RP and metritis 3-4 times. Other predisponing or associated factors include: twinning, short dry periods, various stressors, heredity, MF, exposure to toxins,

mycotoxins, nitrates, abnormally low prostaglandin F2 concentrations in placentomes, other atypical peripartum profiles of steroid, pituitary and adrenal hormones. Immunosuppression in the peripartum period has been implicated as a contributing factor of RP.

Retained placenta as a risk factor for metritis is very much supported in the literature, and a probable biological explanation could be that retained placental membranes pose a perfect media for bacterial growth. However, when retained placenta was not present, large-breed cows had significantly higher odds of metritis than Jersey cows. This would be expected, because Jersey cows generally have an easier calving (less exposed to farmer-assisted calving), and thereby are less prone to trauma to the uterus. Because trauma to the uterine wall can be a source of infection (Fishwick, 1997), this could explain the difference in odds of metritis between the breeds when retained placenta was not present. Path analyses showed that multiparous cows having MF were two times more likely to have RP and metritis (Erb et al., 1981).

Nutritional factors of RP are primarily due to the diet fed the last 6-8 weeks before calving [Curtis et al., 1985]. Extreme deficiency of dietary energy, protein or both can result in RP. Cows fed diets for the entire dry period low in dietary crude protein (8%) had a higher incidence (50%) of retained placenta compared with cows fed 15% crude protein (20% incidence). Fat cow syndrome also frequently is associated with increased incidence of retained placenta and metritis. The rate of retained placenta was associated with imbalances in Ca and P metabolism. Cows with retained placenta had lower antioxidants in blood plasma during the 2 weeks before calving than cows without retained placenta. Supplementation of diets with antioxidants (vitamin E and selenium) to meet the requirements is crucial during the periparturient period. The retained placenta could be significantly reduced when the diet contained at least 0.12 mg/kg of Se and 1000 IU of dietary vitamin E per cow/day**.**

CONCLUSION

Reproduction is one of the key targets in dairy production. Many dairy herds do not achieve appropriate reproductive performance causing subsequent economic loss. Reproductive performance is directly related to health and immunity in the weeks immediately before and after calving. Reproductive diseases including cystic ovarian follicles, anoestrus, retained placenta, endometritis and metritis present a great problem in dairy cow's management.

Evaluation of the effect of oxidative stress on cows´ fertility represents a significant gap in our knowledge about reproduction suggesting a more research in this area to improve reproductive performance of dairy herds. Clear understanding of pathophysiology of negative energy balance and oxidative stress could contribute to better approach to reproductive management of dairy cows avoiding reproductive diseases as much as possible.

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Chapter 3

PRODUCTION DISEASES IN DAIRY HERDS: MONITORING TRANSITION COWS

Hesam A. Seifi*
Department of Clinical Sciences,
School of Veterinary Medicine, Ferdowsi University of Mashhad,
Mashhad, Iran

ABSTRACT

The production diseases of the dairy cow are manifestation of the cow's inability to cope with the metabolic demands of high milk production. While traditionally regarded as encompassing the significant metabolic disorders of dairy cows (hypocalcemia, hypomagnesemia, and ketosis), the term "production disease" has been broadened to include conditions such as retained placenta, displacement of abomasums and laminitis. Most production diseases occur during the first weeks of lactation. The etiology of these diseases can be traced back to insults that occurred during transition period. Grummer (1995) defined the transition period as 3 weeks pre-partum to 3 weeks after parturition. It is a period marked by changes in endocrine status to accommodate parturition and lactogenesis.

Over the past 20 years, our understanding of 'transition cow' metabolism and its relationship to the pathogenesis of peri-parturient

* School of Veterinary Medicine, Ferdowsi Universityof Mashhad, P.O.Box:91775-1793, Mashhad-Iran. E-mail: haseifi@um.ac.ir

disease has greatly increased. There is now significant interest in the critical role peri-parturient disease plays in dairy farm profitability, and in how the risks of such disease and attendant animal culling can be predicted. The risk of many peri-partum diseases of dairy cows is influenced considerably by the nutritional and metabolic status of the animal and in particular, poor adaptation to negative energy balance, is associated with an increased risk of subsequent disease.

Some routinely measured biochemical analytes can be used to predict the development of production diseases in dairy cows. Specific analytes that are either high or low relative to defined reference or 'cut-point' values before calving or immediately post-partum can predict the risk of specific or collective peri-parturient disease events. It was shown that measurement of nonesterified fatty acids (NEFA), β-hydroxy butyrate (BHBA) and calcium concentrations in the first and second week post-partum may provide useful supplementary information for herd health monitoring and culling risk. Hyperketonemia in the first week of lactation is an important risk factor for the subsequent diagnosis of dispalsed abomasums, clinical ketosis and metritis. Additionally, there was a relationship between the concentrations of NEFA at calving and the incidence of certain periparturient diseases. Researchers detected a greater decrease in serum cholesterol concentration and increase in NEFA concentration during the transition period in cows developed retained placenta.

Whole herd interpretation is best made by calculating a proportion of cows above or below a threshold value.

INTRODUCTION

During the past 100 years the US dairy industry has experienced a broad array of major changes including: a sharp reduction in total cow numbers, a near six-fold increase in average production per cow and substantially greater total annual milk production, a truly remarkable biological phenomenon. Dairy cow numbers began in twentieth century at about 17 million, peaked in 1944 at 25.6 million and today are at about 9.1 million. Annual production per cow began last century at just over 3000 lbs, increased to 5314 lbs in 1950 and today is above 17000 (Coppock, 1999). Most of this large increase in production occurred since 1950. The major factors responsible for this large increase are a combination of improved genetics, nutrition and management. Increased genetic merit for milk accounted for 36% of this gain, and the remainder or 64% of the gain can be attributed to some combination of nutrition and management (Everett, 1999).

In addition, approximately 3% of the 70000 dairy herds in the United States have over 500 cows, with an average herd size of >1100 cows, and these herds account for 36% of the milk supply in 2002. It is projected that in 2020, large herds (>500 cows) with an average size of almost 1900 cows will account for 23% of the 15000 herds that remain, and will produce 85% of the milk supply. Large dairies have offered, and will increasingly offer, unique challenges and opportunities for advances in disease prevention(LeBlanc *et al.*, 2006). Metabolic disease incidence typically increases as milk production increases and as herds become larger (Oetzel, 2004).

The objective of this chapter is to cast light upon the recent advances and findings in monitoring health and disease of transition cow, by blood metabolites and minerals measurement.

PRODUCTION DISEASES

The term "production disease" includes all conditions which are attributable to an imbalance between the rates of input of dietary nutrients and the output of production (Radostits *et al.*, 2007). The production diseases of the dairy cow are manifestation of the cow's inability to cope with the metabolic demands of high milk production. While traditionally regarded as encompassing the significant metabolic disorders of dairy cows (hypocalcemia, hypomagnesemia, and ketosis), the term "production disease" has been broadened to include conditions such as retained placenta, displacement of abomasums and laminitis (Mulligan and Doherty, 2008). The aetiology of those metabolic diseases can be traced back to insults that occurred during transition period.

TRANSITION PERIOD

Grummer defined the transition period as 3 weeks pre-partum to 3 weeks after parturition (Grummer, 1995). In an excellent review which still represents the state of art in this area, Drackley referred to transition period as the final frontier (Drackley, 1999). The transition from the dry pregnant, nonlactating state to the nonpregnant, lactating state is too often a disasterous experience for the cow (Goff and Horst, 1997) and it is a strong determinant of the health and performance success of the cow through the full lactation

(Duffield *et al.*, 2009). It is a period marked by changes in endocrine status to accommodate parturition and lactogenesis. These changes, influence tissue metabolism and nutrient utilization. Parturition and the onset of lactation impose tremendous physiological challenges to the homeostatic mechanisms of the cow (Goff and Horst, 1997). A reduction in feed intake is initiated during the pre-partum transition period, yet nutrient demands for support of conceptus growth and initiation of milk synthesis are increasing (Grummer, 1995). The period is characterized by negative energy balance, fat mobilization, and elevation of circulating non-esterified fatty acids and ketone bodies (Ingvartsen and Andersen, 2000). Most infectious and metabolic disorders occur during this time. Milk fever, ketosis, retained placenta membranes, metritis, and displaced abomasums primarily impact cows during the first 3 weeks of lactation (Drackley, 1999).

Over the past 20 years, our understanding of 'transition cow' metabolism and its relationship to the pathogenesis of peri-parturient disease has greatly increased. There is now significant interest in the critical role peri-parturient disease plays in dairy farm profitability, and in how the risks of such disease and attendant animal culling can be predicted (Van Saun, 2006). The risk of many peri-partum diseases of dairy cows is influenced considerably by the nutritional and metabolic status of the animal and in particular, poor adaptation to negative energy balance, is associated with an increased risk of subsequent disease (Herdt, 2000b).

BLOOD PROFILES

Blood tests from individual animals are routinely used to diagnose disease problems in dairy cattle. The Compton Metabolic Profile Test (MPT) first introduced in the early 1970s (Payne *et al.*, 1970). A metabolic profile is defined as a series of specific analytic tests run in combination and used as a herd based, rather than individual based, diagnostic aid. The original intent of the MPT was to monitor metabolic health of the herd, help diagnose metabolic problems and production diseases(Van Saun, 2009). Blood profiles have frequently been used to assess nutritional status of cows in the transition period(Ingraham and Kappel, 1988; Kida, 2002). Such profiles have also been used to monitor herd health and to find subclinical disease,and to predict risk of production diseases (Oetzel, 2004; Macrae *et al.*, 2006).

Different parameters are required when determining the risk of sub-clinical or clinical disease than when making a diagnosis of disease. Some

routinely measured biochemical analytes can be used to predict the development of production diseases in dairy cows. Specific analytes that are either high or low relative to defined reference or 'cut-point' values before calving or immediately post-partum can predict the risk of specific or collective peri-parturient disease events (Van Saun, 2009). Elevated pre-fresh non-esterified fatty acid (NEFA) concentrations (≥ 0.4 mEq/L) and post-fresh ß-hydroxybutyrate (BHBA) concentrations (≥ 1200 µmol/L) are recognized risk factors for ketosis and left-displacement of the abomasum, respectively (Geishauser *et al.*, 2000a; Duffield, 2004; Oetzel, 2004; LeBlanc *et al.*, 2005) .

Whole herd interpretation is best made by calculating a proportion of cows above a threshold value.

NEGATIVE ENERGY BALANCE

Negative energy balance (NEB) is prevalent in dairy cows during the first 2 to 6 weeks of lactation because feed intake does not keep pace with the rapid increase in energy demands for milk production. Milk production requires large amounts of carbohydrate for the synthesis of lactose. Lactational carbohydrate demands are met in ruminants by synthesis of glucose, referred to as gluconeogenesis. A major substrate for gluconeogenesis in ruminants is propoinic acid, one of the volatile fatty acids arising from rumen fermentation. Although propoinic acid is efficiently converted to glucose, there is still a net loss of dietary carbohydrate associated with rumen fermentation. This is because propoinic acid accounts for no more than a third of the total energy available from fermented carbohydrate, the rest being represented by acetic and butyric acids. These latter two acids cannot support gluconeogenesis (Herdt, 2000a). Adipose tissue represents the body's reserve of stored energy. Negative energy balance results in the release of large amounts of NEFA from adipose tissue. Within the liver, NEFAs can be metabolized to ketone bodies or reesterified for the production of triglycerides. When the demand for the glucose outstrips the capacity of the liver for gluconeogenesis, the pathways are maximally stimulated, but the supply of glucose precursors is insufficient to permit maximal glucose production. This results in high rates of ketogenesis and high blood ketone bodies. This hypoglycaemic, classic type of ketosis which generally occurs 3-6 weeks after calving called "Type I ketosis" (Holtenius and Holtenius, 1996). Type II ketosis or fatty liver occurs when large amounts of NEFAs are delivered to the liver, but gluconeogenesis and ketogenesis are not maximally stimulated. Non-esterified fatty acids not used

for ketone body formation and esterified, forming triglyceride. The capacity of cows for mobilization of triglyceride from their liver is easily overwhelmed when blood NEFA concentrations are high and ketone body synthesis rates are relatively moderate. When this occurs, fatty liver develops (Holtenius and Holtenius, 1996; Herdt, 2000a). This manifestation of NEB mostly appears earlier in the lactation and in many cases in combination with other problems as metritis, mastitis, laminitis or other hoof diseases, etc. (Holtenius and Holtenius, 1996). Non-esterified fatty acids, β-hydroxybutyric acid and glucose are considered as energy indicators.

The most commonly identified nutritional constraint was related to energy balance, with 70.4 per cent of cows 10 to 20 days after calving having one or more energy metabolites outside the optimum range (Macrae *et al.*, 2006).

NEFA- Non-esterified fatty acids are sensitive indicators of energy balance. They are useful for monitoring energy status of dry cows in last month of gestation. NEFA concentrations normally increase at calving and reach peak levels on first 10 days post-partum and decrease thereafter (Seifi *et al.*, 2007b). This increase is a result of mobilization of NEFA from adipose tissue to provide energy for parturition and lactogenesis (Vazquez-Anon *et al.*, 1994; Grum *et al.*, 1996). NEFA reflects the magnitude of fat mobilization from fat stores in response to negative energy balance (LeBlanc, 2006). The gradual increase of plasma NEFA during the final days pre-partum may be explained by the gradual depression of dry matter intake (DMI) observed during this time (Bertics *et al.*, 1992). However, DMI was not the only factor influencing pre-partum adipose tissue mobilization (Vazquez-Anon *et al.*, 1994). Prior to parturition, hormone concentrations change to promote gluconeogenesis and mobilization of adipose tissue to provide enough energy to the developing mammary gland and to the limited extent, to the fetus (Herdt, 1988).

NEFA levels of pre-fresh period (at day 8 before calving) had a positive correlation with triglyceride ($r = 0.74$), BHBA ($r = 0.45$) and AST ($r = 0.66$) of post-calving days (day 7 after parturition). However, the correlations of NEFA with other energy metabolites at day 8 post-partum were weaker (triglyceride, $r = 0.52$ and AST, $r = 0.45$) (Seifi *et al.*, 2007b). This finding indicates that NEFA testing at last days of dry period is a reliable predictor of fat mobilization and energy status of transition period.

Elevated NEFA concentrations in pre-fresh cows are associated with high risk for fatty liver, ketosis (Kaneene *et al.*, 1997), and displaced abomasums after calving (Cameron *et al.*, 1998; LeBlanc *et al.*, 2005). Melendez and colleagues found there was a relationship between the concentrations of

NEFAs at calving and the incidence of certain periparturient diseases. Cows with NEFA concentrations ≥ 1.2 mmol/L had a higher incidence of clinical mastitis and milk fever than that of cows with values <1.2 mmol/L (Melendez *et al.*, 2009).

BHBA- β-hydroxybutyric acid, one of the ketone bodies, is another parameter useful in assessing energy balance. Serum BHBA concentrations are higher post-partum than pre-partum (Bertics *et al.*, 1992; Vazquez-Anon *et al.*, 1994; Cavestany *et al.*, 2005; Seifi *et al.*, 2007b), because of the high-energy demands associated with the onset of lactation (Vazquez-Anon *et al.*, 1994). BHBA concentrations had significant correlations with triglyceride , NEFA and AST at post-partum period (Seifi *et al.*, 2007b).

Hyperketonemia in the first week of lactation is an important risk factor for the subsequent diagnosis of dispalsed abomasums, clinical ketosis and metritis (Duffield *et al.*, 2009). Increased health risk and reduced milk production appear to start between a threshold of 1200 to 1400 μmol/L of serum BHBA during the first week post-calving (Duffield *et al.*, 2009). Before calving, BHBA concentrations are not predictive for disease risk (Van Saun, 2004). BHBA can come from dietary sources (poorly fermented silage) and not reflect aberrant metabolism (Van Saun, 2009).

Glucose- Blood glucose concentration, as an independent test, is not good indicator of energy status as a result of tight homeostatic control (Van Saun, 2009). During the 1st week of lactation, plasma glucose concentration decreased 25%, but by the 2nd week in lactation, started to increase. The increase may reflect the recovery of feed intake and improving energy status of the cow (Vazquez-Anon *et al.*, 1994). Although glucose is the primary metabolic fuel, and is absolutely required for vital organ function, fetal growth, and milk production (LeBlanc, 2006), it is an insensitive measure of energy status because it is subject to tight homeostatic regulation (Herdt, 2000a). Serum glucose had no strong correlations with other energy related metabolites during pre- and post-partum (Seifi *et al.*, 2007b).

AST- The activity of AST (Aspartate Aminotransaminase) also was higher for periparturent disorders affected cows than for healthy cow (Dann *et al.*, 2005). It was shown that Increased activity of AST was associated with subsequent incidence of fatty liver and abomasal displacement(Herdt, 1988; Geishauser *et al.*, 1997).

AST activity was the lowest at day 22 before calving and highest at day 21 post-partum. In addition, AST significantly correlated to triglyceride (r = 0.61), BHBA (r = 0.54), NEFA (r = 0.47), cholesterol (r = 0.44), and glucose (r = 0.3) at day 8 post-partum. In addition, AST significantly correlated to

BHBA and NEFA (r = 0.51 and 0.73, respectively) at day 21 post-partum. It seems AST is the most correlated with other energy indicators at transition period(Seifi *et al.*, 2007b). AST is the more correlated hepatic enzyme with fatty infiltration of liver in dairy cows (Herdt, 1988), and AST activities greater than 100 u/L could be used as diagnostic aid for fatty infiltration (Geishauser *et al.*, 1997).

Cholesterol- The significance of blood cholesterol concentrations at transition period is somewhat controversial. Some authors associate a rise in cholesterol to a better energy balance or fat intake(Wittwer *et al.*, 1987), while others postulate that it would be the result of energy deficiency(Bruss, 1997). Cholesterol concentration decreases during dry period. It was low before parturition, but increased sharply at day 21 post-partum (Seifi *et al.*, 2007b). In addition, cholesterol had a negative correlation with BHBA at day 21 post-partum (Seifi *et al.*, 2007b).

BODY PROTEIN STATUS

The serum concentration of urea nitrogen (BUN or SUN) is related to the breakdown of effective rumen-degradable protein in the rumen, and its conversion into microbial protein. It is therefore related to short-term intakes of rumen degradable protein from the diet, and also reflects the balance between degradable protein and the energy available to the rumen microbes. Low concentrations may reflect the fact that either the ration is deficient in degradable protein or that the ration has adequate protein but cows are not sufficient quantities of it (Macrae *et al.*, 2006). Serum albumin concentrations are indicative of liver function and long-term protein status (Macrae *et al.*, 2006; Radostits *et al.*, 2007).

BUN -Serum BUN was lowest for dry cows, higher in early lactation and subsequently higher during the lactating pregnant period (Peterson and Waldern, 1981; Seifi *et al.*, 2005b). The increase of serum BUN after parturition is associated with the increase in feed intake (Bauchart, 1992). It was shown that 16 per cent of the cows in early lactation had plasma urea nitrogen concentrations below 9.79 mg/dL. The highest percentage of problems was observed in the dry cows within 10 days to calving, 20.5 per cent of which had low BUN concentrations, suggesting their impaired rumen function, with potentially important consequences for the efficient digestion and utilization of the ration in early lactation (Macrae *et al.*, 2006).

The effect of high urea nitrogen on fertility remains controversial. However, it was stated that fertility decreases when serum BUN reach 20 mg/dL or more (Ferguson *et al.*, 1993).

Albumin –Albumin is a protein synthesized in the liver. Low levels reflect poor liver health or a poor supply of amino acids from the diet. Low levels will only appear after prolonged and severe underfeeding of protein (Whitaker, 2000).There was a tendency for cows to have lower concentrations of serum albumin shortly after calving than at any other stage of the lactational cycle (Seifi *et al.*, 2007b). The albumin decrease at parturition was associated with the decrease in calcium concentrations. This could be because one fraction of the total pool of calcium is linked to albumin and depends partly on its concentration (Goff, 2000; Seifi *et al.*, 2005b).

Globulin – High concentrations of globulin can help to identify cows with chronic inflammatory problems, such as mastitis, metritis and lameness (Macrae *et al.*, 2006).

MACRO-MINERALS BALANCES

Macro-minerals calcium, phosphorus, potassium, magnesium, sodium, chloride and sulfur are of extreme interest relative to their role in milk fever, alert downer cow syndrome and weak cow syndrome. However, most of these minerals are tightly regulated through a variety of homeostatic processes. Phosphorus, magnesium and sulfur are minerals in which blood concentrations are somewhat sensitive to dietary intake(Van Saun, 2006). Milk fever and subclinical hypocalcemia (total blood calcium $\leq$ 2.0 mmol/L) are most important macro-mineral disorders that affect transition dairy cows (Mulligan *et al.*, 2006). On average 5-10% of dairy cows succumb to clinical milk fever (Houe *et al.*, 2001), with the incidence rate of subclinical hypocalcemia has been recorded at 30-40% on the day of calving (Roche, 2003). The hypocalcemia is related to increased incidences of mastitis, dystocia, prolapsed uterine, retained placenta, endometritis, slower uterine involution and delayed first ovulation after calving, ketosis and displacement of abomasum (Mulligan *et al.*, 2006).

Electrolytes sodium, chloride and potassium are altered when renal or digestive function is compromised (Van Saun, 2009). Homeostatic control of sodium and potassium is too strong to allow measurement in blood to be a reliable indicator of dietary intake(Whitaker, 2000), so electrolytes levels are only changed in extreme dietary deficiency states, (Van Saun, 2009)

Calcium – Monitoring of dairy herds for subclinical hypocalcemia involves blood-sampling cows about 12-24 hour after calving. The total blood calcium concentration of 2.0 mmol/L (8 mg/dL) has been suggested as a target for subclinical hypocalcemia (Oetzel, 2004). Either pre- or post-partum cows with serum total calcium below 2.0 mmol/L were four times more likely to have post-partum disease problems (Van Saun, 2006). Parturient hypocalcemia is a risk factor for subsequent displaced abomasum (Massey *et al*., 1993). The significant association of calcium concentration of less than 2.3 mmol/L at first week after calving with subsequent risk of abomasal displacement and culling was reported. The odds of the development of displacement of abomasum were 5.1 times greater in cows with serum calcium concentrations $\leq$ 2.3 mmol/L in the first week post-partum. Furthermore, The odds of culling in early lactation were 2.4 and 5.3 times greater in cows with serum calcium concentrations $\leq$ 2.2 and $\leq$ 2.3 mmol/L in the first and second weeks after calving, respectively (Seifi *et al.*, 2010).

Homeostatic control of the level of calcium in the blood is so strong that variations are small and do not reflect dietary intake at all (Whitaker, 2000).

Inorganic Phosphorus – Excessive dietary phosphorus in dry period has been associated with high rates of milk fever and downer cows (Weaver, 1987). In addition, high concentration of dietary phosphorus can cause reproductive problems (Hoedemaker *et al.*, 1992). Hyperphosphatemia was seen in cows fed excessive dietary phosphorus. High levels of serum inorganic phosphorus was also associated with reproductive problems (Seifi and Bazargani, 2003). Low levels of serum inorganic phosphorus were also related to decreased reproductive performance (Seifi *et al.*, 2005a). Furthermore, hypophosphatemia can be a contributory cause of the downer cow syndrome (Whitaker, 2000).

Magnesium – Serum magnesium concentrations vary with the intake of dietary magnesium, and low concentrations were recorded most often in freshly calved cows (Macrae *et al.*, 2006). Subclinical hypomagnesemia can lead to reductions in dry matter intake and milk yield (Macrae *et al.*, 2006). In addition, subclinical hypomagnesemia during the dry period has been associated with milk fever, owing to the requirement for magnesium in calcium homeostasis (Contreras *et al.*, 1982; Sansom *et al.*, 1983).

Sodium– Pre- and post-partum sodium concentrations were also highly associated with post-partum disease risk(Van Saun, 2006). Serum sodium has the least variable in the profile test (Payne *et al.*, 1970), But there were some reports indicated that low serum sodium concentrations resulted in pica(Payne *et al.*, 1970; Seifi and Bazargani, 2003). Payne *et al.* commenting on Pica,

stated that cows do drink each other's urine and lick everything in order to find meager salt supplies (Payne *et al.*, 1970). Similar Pica symptoms and poor growth in beef cattle was observed in low sodium pasture (Murphy and Plasto, 1972). In a report, the average sodium level of 110 mEq/L was associated with pica and urine licking (Seifi and Bazargani, 2003).

Potassium– Recently hypokalemia syndrome has been defined in dairy cows. Most of the cases were diagnosed secondarily to gastero-intestinal diseases particularly ketosis. Reduced potassium intake, intracellular shifting of potassium subsequent to metabolic alkalosis and hyperglycemia, kaluresis due to hyperglycemic osmotic diuresis, and increased potassium loss due to the mineralocortocoid effects of exogenously administered corticosteroids are all potential contributory factors to the development of clinically significant hypokalemia in the chronically ketotic cow (Peek *et al.*, 2003).

MONITORING PRODUCTION DISEASES

Specific analytes that are either high or low relative to defined reference or 'cut-point' values before calving or immediately post-partum can predict the risk of specific or collective peri-parturient disease events (Van Saun, 2009). Measurement of NEFA, BHBA, and calcium concentrations in the first and second week post-partum may provide useful supplementary information for herd health monitoring (Seifi *et al.*, 2010).

Cows with higher concentrations of NEFAs at calving will experience a greater incidence of peri-parturient diseases compared with that in cows with lower concentrations of NEFAs (Melendez *et al.*, 2009).

Elevated pre-fresh non-esterified fatty acid (NEFA) concentrations (≥ 0.4 mEq/L) and post-fresh ß-hydroxybutyrate (BHBA) concentrations (≥ 1200 µmol/L) are recognized risk factors for ketosis and left-displacement of the abomasum, respectively (Geishauser *et al.*, 2000a; Duffield, 2004; Oetzel, 2004; LeBlanc *et al.*, 2005).

Hyperketonemia in the first week of lactation is an important risk factor for the subsequent diagnosis of displaced abomasums, clinical ketosis and metritis (Duffield *et al.*, 2009).

Retained Placenta - Pre-partum energy metabolism is an element that contributes to the occurrence of retained placenta. Researchers detected a greater decrease in serum cholesterol concentration and increase in NEFA and BHBA concentrations during the transition period in cows developed retained placenta (Kaneene *et al.*, 1997; Seifi *et al.*, 2007a; Seifi *et al.*, 2007b; Quiroz-

Rocha *et al.*, 2009). In the study of Quiroz-Rocha *et al.*, despite the significant association of cholesterol and NEFA with subsequent development of retained placenta, serum concentration of neither NEFA nor cholesterol was a strong diagnostic test for prediction of retained placenta (Quiroz-Rocha *et al.*, 2009). As serum concentration of cholesterol or NEFA increased 0.1 mmol/L in the last week prior to parturition, the odds of developing retained placenta increased by 5% (Quiroz-Rocha *et al.*, 2009).

Furthermore, the concentrations of serum albumin and BUN were also significantly lower in retained placenta affected cows than non-affected ones after parturition (Seifi *et al.*, 2007a). However, these findings did not reveal a cause and effect relationship with respect to the role of negative energy balance and protein status as possible risk factors for retained placenta.

Abomasal displacement –Several studies have shown that the severity of peri-partum negative energy balance, reflected by NEFA and BHBA concentrations, is a key element in the etiology of left displacement of abomasum (Cameron *et al.*, 1998; LeBlanc *et al.*, 2005; Duffield *et al.*, 2009; Seifi *et al.*, 2010). NEFA and BHBA provide better insight into metabolic function with respect to development of left displacement of abomasum.

Cows with plasma NEFA >0.3 mEq/L between 3 and 35 days before calving were twice as likely to subsequently have a displaced abomasums (Cameron *et al.*, 1998). Other studies strengthen and refine the application of pre-partum NEFA measurement for assessment of the risk of left displacement of abomasum. LeBlanc *et al.* (2005) found a stronger association of NEFA concentrations (Odds ratio = 3.6) at the cut-point of ≥ 0.5 mEq/L.

Subclinical ketosis and serum AST activity in the first 2 weeks post-partum were associated with increased risk of left displacement of abomasums (Geishauser *et al.*, 2000b). The findings of several studies suggest that subclinical ketosis is a risk factor for displacement of abomasum (Geishauser *et al.*, 2000b; LeBlanc *et al.*, 2005; Duffield *et al.*, 2009; Seifi *et al.*, 2010). The findings of the study of Seifi *et al.* reinforce the predictive association of elevated concentrations of BHBA with the risk of displacement of abomasum. In the first week after calving, cows with BHBA concentrations ≥ 1000 μmol/L were 13.6 times more likely to develop displacement of abomasum (Seifi *et al.*, 2010) .

In a large study involving more than 1000 cows in 25 herds, AST and BHBA were significantly increased in the first and second week after calving in cows diagnosed with displaced abomasum 1 to 3 weeks later as compared with control cows. If serum AST activity in the first week post-partum was 101 u/L or greater, the odds were three to one (compared to less than 101 u/L)

that displacement of abomasum would be diagnosed 1 to 3 weeks later. In the second week post-partum the odds ratio was eight to one (Geishauser *et al.*, 1997).

In those cows that subsequently developed displacement of abomasum, serum glucose concentration was significantly decreased in the second week after calving but not in the first week, compared to cows that did not develop displacement of abomasum (Geishauser *et al.*, 1998). In a recent study, there was no findings regarding glucose association to displacement of abomasum (Seifi *et al.*, 2010).

Low levels of cholesterol were reported in some Swedish herds with the history of abomasum displacement and ketosis incidence(Stengarde *et al.*, 2008). The potential usefulness of cholesterol in prediction of Abomasal displacement needs further investigation.

The significant association of calcium with the subsequent risk of displacement of abomasum is reported. It is generally considered that hypocalcemia may reduce the tonus of the abomasum and result in an increase in the accumulation of gas (Massey *et al.*, 1993; Doll *et al.*, 2009). The study of Seifi *et al*. indicated that cows with total serum calcium concentration within the lower half of the normal range (< 2.2 mmol/L) in the first two weeks post-partum were at greater risk of both developing displacement of abomasum and of being culled within 60 days in milk (Seifi *et al.*, 2010). The odds of the development of displacement of abomasum were 5.1 times greater in cows with serum calcium concentrations ≤ 2.3 mmol/L in the first week post-partum (Seifi *et al.*, 2010). However, the findings of a previous study indicated that serum calcium is of limited value for prediction of displacement of abomasum (Geishauser *et al.*, 1998).

Ketosis – The optimum BHBA and NEFA cut-points based on maximum total sensitivity and specificity for clinical ketosis was 1200 μmol/L and 1 mmol/L in the first week post-partum, respectively. Cows with BHBA concentrations ≥ 1200 μmol/L were 4.7 times more likely to develop clinical ketosis. The odds of a diagnosis of clinical ketosis were 6.3 times greater in cows with serum NEFA concentrations of ≥1.0 mmol/L in the first week after calving (Seifi *et al.*, 2010).

Mastitis -Melendez *et al*. found there was a relationship between the concentrations of NEFAs at calving and the incidence of certain peri-parturient diseases. Cows with NEFA concentrations ≥ 1.2 mmol/L had a higher incidence of clinical mastitis than that of cows with values <1.2 mmol/L (Melendez *et al.*, 2009). However, week 1 post-partum BHBA was not associated with subsequent diagnosis of clinical mastitis (Duffield *et al.*,

2009). The authors discussed the lack of an association due to both underreporting of mastitis and a wide risk interval (up to 95 days in milk).

Milk Fever –Assessment of calcium concentrations around the time of calving is a useful indicator of how well the calcium regulatory system is working and potential for clinical or subclinical hypocalcemia problems (Oetzel, 2004).

Cows with NEFA concentrations ≥ 1.2 mmol/L at calving had a higher incidence of milk fever than that of cows with values <1.2 mmol/L (Melendez *et al.*, 2009). Per each 0.1 mEq/L increment of NEAFs at calving, the odds ratio of experiencing milk fever was 1.10 times. The association between high NEFAs and milk fever is more difficult to explain. There is no cause and effect relationship. Cows with hypocalcemia probably eat less and mobilized more fat(Melendez *et al.*, 2009).

Subclinical hypomagnesemia during the dry period has been associated with milk fever occurrence (Sansom *et al.*, 1983). Detection of low serum magnesium concentrations pre-partum could be considered as a predictive indicator for hypocalcemia after calving.

Liver Function - Liver function can be assessed through enzymes in blood, such as gamma-glutamyltransferase (GGT), and AST (Reid *et al.*, 1979). Testing of blood hepatic enzyme activities is poorly correlated with hepatic triacylglycerol (TAG) with the exception of elevated AST (Reid *et al.*, 1983). AST was significantly increased in the fatty liver and ketosis groups compared with the control group(Steen *et al.*, 1997). This was the enzyme that correlated best with reduced hepatic function in fatty liver disease in other investigations (Herdt, 1988) and has been selected in herd monitoring programs for the occurrence of fatty liver (Reid *et al.*, 1983). In addition, it was shown that fatty liver affected cows were characterized by high free fatty acid levels and low cholesterol concentrations, too (Steen *et al.*, 1997). Calculating the NEFA-to-cholesterol ratio to assess the liver's ability to export incoming NEFA has been advocated. Calculated NEFA-to-cholesterol ratio was predictive for post-partum disease in the close-up dry and fresh cows (Van Saun, 2009).

Infertility - The multifactorial nature of infertility is an important reason why investigators have met varying success when they turned to profile tests as a potential panacea for the diagnosis of all reproductive problems. Many investigators link infertility with abnormalities of energy, protein and mineral status. There have been experiments, which indicate low glucose(McClure, 1968; McClure, 1970), increased ketone bodies (Andersson and Emanuelsson, 1985), decreased serum concentration of cholesterol(Seifi *et al.*, 2005a), low serum albumin(Rowlands *et al.*, 1980), high blood urea(Ferguson *et al.*, 1988),

high or low serum phosphorus(Hewett, 1974; Pugh *et al.*, 1985; Seifi *et al.*, 2005a), other mineral imbalances(Pugh *et al.*, 1985; Seifi *et al.*, 2005a), reduced liver function (Reid *et al.*, 1979), energy deficit(Villa-Godoy *et al.*, 1988; Butler and Smith, 1989), and overfeeding with protein (Hewett, 1974; Chalupa, 1984) can all be related to fertility problems.

Protein nutrition can affect reproduction through toxic effects of ammonia and its metabolites on gametes and early embryos, through deficiencies of amino acids, and by exacerbations of negative balances of energy (Ferguson and Chalupa, 1989).

An excessive loss of body condition during the transition period is a major risk factor for health and fertility disorders (Roche *et al.*, 2007). Therefore, increased concentrations of NEFA in this period could be resulted in low fertility.

Metritis – The association between increased ketones and subsequent diagnosis of metritis was reported (Dohoo and Martin, 1984; Duffield *et al.*, 2009). Cows with BHBA concentrations more than 1200 μmol/L in the first week post-partum were 3.35 times greater at the risk of developing metritis (Duffield *et al.*, 2009). An indirect effect of hyperketonemia on immune function and decreased DMI are most likely explanations of this finding (Suriyasathaporn *et al.*, 2000; Huzzey *et al.*, 2007; Duffield *et al.*, 2009). Similar effects for ketones on subclinical endometritis was described (Hammon *et al.*, 2006).

Culling - NEFA and calcium concentrations at week one and two post-calving were associated with subsequent culling during the early lactation period. The odds of culling in early lactation were 2.4 and 5.3 times greater in cows with serum calcium concentrations $\leq$ 2.2 and $\leq$ 2.3 mmol/L in the first and second week after calving, respectively. In addition, cows with NEFA concentrations $\geq$ 1.0 mmol/L in the first week post-partum, were 3.6 times more likely to be culled within the next two months (Seifi *et al.*, 2010). Van Saun *et al.* reported that healthy cows had higher calcium concentrations pre- and post-partum compared to animals which exhibited one or more disease conditions. Irrespective of time relative to calving, cows with serum calcium concentrations < 2.0 mmol/L pre- or post-partum were at 3.8 and 4.0 times greater risk of developing post-partum disease, respectively (Van Saun *et al.*, 2005).

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Chapter 4

INCREASING THE VALUE IN DAIRY CHAINS MAINLY SUPPLIED BY SMALL SCALE FARMS: CASE STUDY FROM MOROCCO

Sraïri Mohamed Taher
Hassan II Agronomy and Veterinary Medicine Institute,
Madinate Al Irfane, 10 101, Rabat, Morocco

ABSTRACT

Global demand of dairy products and its recent developmentmay put the world supply at risk. To fulfill growing needs, it is widely accepted that a "Livestock Revolution" will be required worldwide. This trend will have to be carried in developing countries, particularly by targeting small scale farms which represent the main actors in animal products' supply chains. However, intervention in such farms requires specific means which consider their characteristics: low cost laborof limited know-how, land, water and financial resources' scarcity. There is an urgent need to reassess the agricultural strategies and the economic managementin these farms, before implementing adapted intervention policies. In this article, the specific case of dairy cattleproduction in Morocco and its possible upgrading to reach international standards is reviewed. The context of cattle farming in this country is presented first. Indeed, there is a fragmented offer with numerous batches of relatively limited volumes delivered every day. This has induced the emergence of milk collection co-operatives. Milk deliveries are thus organized in a two stage way (from farms to collection centers, then to milk plants), which constitutes

a significant constraint to improve quality. Second, intervention possibilities in such chains are presented. A research programwas designed to enhance milk yield and quality. Itsprimary objective was to achieve a diagnosis of dairy production performances (milk yield, raw margin per cow, etc.) in a sample of representative farms. It also allowed characterizing water productivity through dual purpose herds (both milk and meat), as water scarcity represents a priority issue in the agenda of dairying in Morocco. After the diagnosis, an intervention program was implemented by a targeted follow-up of cows' dietary rations and the use of adequate feed supplementation. Results showed that on-farm intervention by balanced rations calculations provided a sound example to assist small scale farmers improve their performances. Finally, models of milk quality parameters in relation to herds' management practices were conceived. They would allow designing a grid of milk quality payment by an indirect assessment of rearing practices. Such results have yet to be adopted at a large scale by the stakeholders in the dairy chain. That implies generalizing the use of such intervention methods, which may necessitate further negotiations devoted to value chain. This may represent practical solutions to upgrade milk production in Morocco, given its numerous contributors and their constraints.

Keywords: Dairy production, Dual purpose herds, Milk quality, Morocco, Small scale farms, Supply chain, Water productivity

INTRODUCTION

Recent developments in the global food demand have shown a significant increase of prices of animal commodities such as milk and meat (Nin *et al*., 2007). This trend has been worsened by climate change which threaten many livestock systems (Thornton *et al*., 2009) and by evolving consumption habits in major emerging countries, such as China and India, in which milk and meat demand is rapidly expanding (Beghin, 2006). Therefore, there is an urgent need to increase animal products' supply worldwide, to avoid risks on the markets. This can only be achieved by a "Livestock Revolution", which will have to target in priority small scale farms in developing countries, as they represent the vast majority of the actors in animal products' supply chains (Delgado, 2003). Implementing specific policies directed to small scale animal breeding farms requires adapted tools, of which State intervention and proper incentives might be crucial. Indeed, it is with such a policy, namely the

"Operation Flood" that India has become the world's leader in cattle raw milk production (Gautam Dalal and Pathak, 2010). However, these interventions driven by State technical services generate important costs and may not benefit to all categories of small scale farms, because of their scattering. Therefore, many States are currently withdrawing from extension services to farmers, assuming that private operators may be more efficient (Kidd *et al*., 2000). Under the Moroccan context, small scale farms show some obvious assets (low cost family labor and non specialized herds) which allow them benefiting from the dairy activity with steady incomes (Sraïri *et al*., 2009a). However such farms also show structural conditions which induce many limitations to an optimal milk output: scarce arable land and insufficient irrigation water availability combined to a reduced know-how in feeding cattle with balanced rations (Sraïri *et al*., 2009b). All together, these constraints mean that the dairy chain suffers from various drawbacks. These appear in the average milk yield per lactating cow which could be significantly increased whith adapted support programs. These shortfalls also affect milk quality, as the existing structure of the dairy chain, with a fragmented offer (numerous farms with limited volumes delivered every day), imply various consequences on the characterization and the remuneration of milk batches according to their chemical and hygienic quality indicators.

In this article, the upgrading of a dairy chain dominated by small scale farms is presented. First, the Moroccan dairy production sector is presented. Then, two intervention attempts to demonstrate possibilities to upgrade such a dairy chain are illustrated. The first is devoted to supporting small scale cattle farms increasing their milk yield through adapted feeding. The second is related to enhancing milk quality at farm level, by setting up models which link rearing practices to milk quality indicators. Finally, value and limits of these intervention attempts are discussed along with their application at larger scales.

CONTEXT OF THE STUDY: MILK PRODUCTION IN MOROCCO

Located in western North Africa (Figure 1), Morocco is a country characterized mainly by a semi-arid climate and a fast growing human population (15.3 to 30.4 million, from 1975 to 2006). Facing this demographic expansion and changing nutritional habits, the authorities have launched in the

early 1970s, ambitious plans to fulfil the demand for food. For instance, a specific public policy devoted to cattle farming has been implemented to ensure a steady supply of dairy products and beef to the population (Sraïri and Chohin Kuper, 2007).

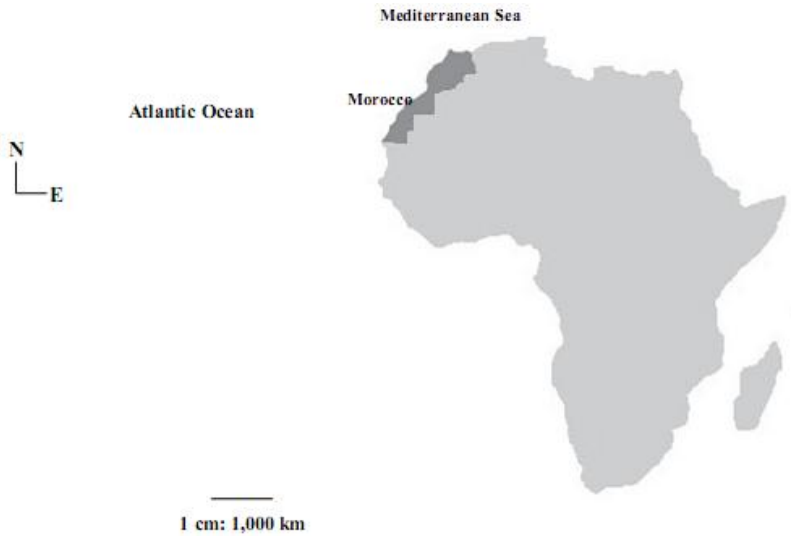


Figure 1. Localization of Morocco in the African continent.

It enabled the annual raw milk output to be increased from 400,000 inearly 1970's to almost 1,600,000 tons in 2008 (MADR, 2009). This mainly happened in large-scale irrigation schemes where, due to erratic rainfall, water is crucial for fodder production: almost 60% of the raw milk volumes originate from these areas although they only represent 15% of arable land (Sraïri et al., 2009a). That policy took in account a dairy cattle sector mainly based on smallholder units (less than five cattle on a farm below 5 ha), as they represent more than 80% of the total number of cattle farms:above 700,000 according to MADR (2009). Smallholder dairy farming in Morocco, is somewhat different from other developing countries, in that it has integrated many aspects of intensive cattle rearing, such as imported breeds (Holstein and Montbéliarde) and their crosses with local strains, and the widespread use of concentrates to feed cows. However, this means dairy production costs are significantly higher than in other African countries (Ndambi and Hemme, 2008). Moreover, dairy performances of small-scale farmers continue to be lower than expected, due mainly to a lack of technical knowledge, particularly

in feeding balanced rations. This induces a limited average profitability per cow, as calf crop is often vital to ensure the economic sustainability of farms (Sraïri et al., 2009b). On another hand, this structure of production also implies that milk quality is often poor. In fact, the fragmented offer is handled by a two stage organization: 1) from farms to co-operative collection centres and 2) then to milk processing plants. This means that it is quite impossible to assess each batch delivered per farm at its actual chemical and hygienic quality, and farmers cannot be paid accordingly (Sraïri et al., 2009c). Therefore, improving milk yield per cow and milk chemical and hygienic quality appear to be crucial to increase the overall income of farms and ensure their sustainability. This would require diffusing adapted dairy farming knowledge, mainly towards small scale farms. Recently, a national plan for agricultural development (Plan Maroc Vert - Green Morocco Plan) has been launched, and it expects to triple the domestic milk output by 2020 (up to 5 million tons annually) from current levels, particularly by promoting large agribusiness farms (MADR, 2008). However, the production structure raises the issue of the contribution of smallholder farms in achieving that objective. Increasing their milk production to respond to market demand should be based on improving milk yield per cow rather than increasing the cow stocking rate, which is already quite high (MADR, 2009). Such an evolution would require a support specifically adapted to the technical and economic situation of these smallholder farms.

A DIAGNOSIS OF A SAMPLE OF IRRIGATED SMALL SCALE DAIRY CATTLE FARMS

A series of follow-up of smallholder cattle farms was achieved in the Tadla irrigated scheme, Centre East of Morocco. This region of 98,000 irrigated ha accounts for 16% of the annual domestic milk output. Raw milk is produced by around 17,000 cattle farms, which mainly rely on irrigated alfalfa (25,000 ha). Some 55,000 lactatingcowsproduce150,000 tons of milkannually with diverse genetic merits: less than25% are local breeds, almost 75% are either pure Holsteinor crosses with local breeds. Nearly all the milk comes from smallholder dual purpose (both milk and meat) farms, as 80% of them cover less than 5 ha of arable land (ORMVAT, 2010).

A sample of six cattle units representative of the overall population was chosen (Table 1), in accordance with a previous work which aimed to establish

a typology of farms in the same region: *i*)specialised dairy farms, *ii*) mixed farming systems (cattle and cash crops) and *iii*) dual-purpose herds (milk and meat). The second and third cattle systems mainly use crossbred cows whereas specialised dairy units imported pure Holstein cows and used artificial insemination (Kuper *et al*., 2006). Then, dairy production practices and performances were characterized, with an emphasis on irrigation water uses, fodderand cattle products (milk and meat) outputs.Results show that the overall performances are widely variable (Table 2). For example, mean annual milk yield per cow was only 2,170 kg, and it varied from 1,650 to 3,400 kg. Indeed, from irrigated fodder to cattle products, evident losses appear, due to inappropriate farming practices, insufficient water availability and imbalanced cattle feeding (Sraïri *et al*., 2009a). As a consequence, the average profitability of dairying is weak (less than 300 US $ per cow including the value of calves), because the cost of production of milk is often higher than farm gate milk price. In fact, only calf crop sales allow a majority of farms to reach an economic equilibrium. Therefore, live animals sales constitute a strategic income for farms, whereas milk allows a steady cash flow to face daily expenses. These trends result on a highly perfectible water productivity through cattle: almost 1.8 m^3 per kg of raw milk and 10.5 m^3 per kg of live weight cattle.

Table 1. Structural characteristics of diagnosed cattle farms in the Tadla irrigated scheme

Farms	1	2	3	4	5	6
Arable land (ha)	5.0	6.3	6.5	1.4	1.6	1.8
Total fodder crops area (ha)	2.7	3.4	2.6	0.8	0.8	1.0
Alfalfa (ha)	2.0	2.0	2.2	0.8	0.8	1.0
Berseem (ha)	0.5	0.7	0.4	-	-	-
Maize (ha)	0.2	-	-	-	-	-
Barley (ha)	-	0.6	-	-	-	-
Herd characteristics						
Lactating cows	6.5	7.0	6.4	2.0	2.0	3.0
Growing cattle	5	7	6	2	2	3
Dairy (D), beef (B) or dual (BD) strategy	BD	BD	BD	D	B	B

Table 2. Technical performances of irrigated cattle farms: from water productivity to profitability

Farms		1	2	3	4	5	6
Milk	Milk output (kg)	14 820	11 900	13 310	6 800	3 800	4 950
	Total water used (m^3)	31 170	25 950	22 200	7 750	5 740	8 970
	Water productivity through milk (m^3/kg of milk)	2.1	2.2	1.7	1.1	1.5	1.8
	Water economic productivity (US \$/m^3)	0.03	0.04	0.10	0.18	0.12	0.12
Meat	Total live weight gain (kg)	2 100	1 740	1 769	430	712	1 290
	Total water used (m^3)	19710	22500	9 980	3 820	6 720	10800
	Water productivity through meat (m^3/kg of milk)	9.4	12.9	5.6	8.9	9.4	8.4
	Water economic productivity (US \$/m^3)	0.27	0.18	0.48	0.34	0.31	0.31
Water economic productivity through cattle (US \$/m^3)		0.12	0.10	0.22	0.23	0.22	0.22

All together, these results imply that urgent measures are needed to ensure the competitiveness of dairying with irrigated fodder in Morocco with regard to other agricultural (fruits, vegetables, etc.) or non agricultural (tourism or industry) activities. This happens in a time where water scarcity is becoming a high priority issue for Morocco, as rhythms of water consumption are not sustainable anymore, because they have resulted in the depletion of many aquifers (Iglesias *et al*., 2007). Therefore, interventions to enhance the performances of dairy producers, mainly the vast majority of smallholder units, would be fruitful (Le Gal *et al*., 2009).

AN INTERVENTION TO INCREASE MILK YIELD PER COW THROUGH CORRECT FEEDING

As the initial diagnosis of dairy farming within irrigated smallholder units revealed improper practices from fodder to cattle production, an intervention was planned to promote lactating cows' milk yield by the adoption of balanced feed rations. Five small-scale cattle farms were chosen and they were visited twice a month from November 2006 to May 2007. This schedule enabled the

cows' true dietary rations to be compared with their total optimal requirements calculated as the sum of their maintenance and potential production needs. The potential energy and protein requirements for milk production were determined using existing models describing variations in daily milk yield during lactation (Wilmink, 1987). These were related to the herds' genetic merit and their monthly lactation stage, which was calculated throughout the study period as shown in Equation (*1*). Calving dates of all the lactating cows were determined during the first visit to the farm in November 2007. Subsequently calving and drying up dates were recorded throughout the monitoring period.

$$Lactation_Stage_j = \sum_{k=1}^{m} Lactation_duration_{k,j} / (Total_Milked_Cows_j \times 30.4) \quad (1)$$

with:

$Lactation_Stage_j$ = lactation stage (in months) for month *j*

$Lactation_duration_{k,j}$ = number of milking days from calving for cow *k* and month *j*

$Total_Milked_Cows_j$ = total number of milked cows for month *j*

The genetic merit of pure Holstein herds was considered to be 7,000 kg of milk annually, whereas an annual milk yield of 4,000 kg was used forHolstein crosseswith local breeds.

During each visit, all the components (i.e. forages and concentrates) of the cows' dietary rations were weighed.The nutritive contents of the rations were determined using feed composition tables. For concentrates, which were mainly imported, the INRA France table was used (Jarrige, 1988), whereas for local fodder and crop by-products (wheat straw and bran, dehydrated beet pulp, etc.), results from Guessous (1991) were used.

At eachvisit, the correspondence between cows' nutritional requirements and the true ration was evaluated. Supplementation was suggested to the farmer when a gap was detected between the dietary ration and potential net energy, ruminally degradable protein (RDP) or metabolizable protein (MP)requirements. The two latter parameters related to the protein status of the diet were determined accordingly to the French system of the PDI - *Protéines Digestibles dans l'Intestin* - (Vérité and Peyraud, 1988). The proposed rations took into account the context of the farm, i.e., the availability of on-farm fodder and the money needed to buy concentrates. The acceptance

of the suggested balanced rations was tested by monitoring cows' average milk yield and noting the farmers' opinions about the nutritional changes that were made.The effects on the profitability of dairy production were also assessed.

Results of the intervention show that the characterisation of lactating cows' dietary rations at the beginning of the study revealed insufficient and imbalanced supply between energy and RDPin all farms. In fact, the main forage supplied is green alfalfa, which provides more protein than energy with respect to the average cow's energy requirements. Table 3 shows an example of the dietary rations used in a farm 1,with pure Holstein cows characterised by a lactation potential of 23 kg of milk daily and an average body weight of 620 kg at the beginning of the monitoring period. It shows an insufficient supply of Dry Matter (DM), which only reached 6 kg of roughage per cow, whereas a Holstein cowcould ingest as much as 15 kg of DM from good quality alfalfa (Castillo *et al*., 2006). And it is also unbalanced, as alfalfa,a leguminous plant, represents the bulk of the initial roughage intake, leading to a relative excess of RDP whereas net energy is lacking. The amount of both energy and MP supplied were thus insufficient to cover total requirements (i.e. maintenance and potential production). For that reason, this dietary ration was not suitable to reach the lactation potential of the herd.

Table 3. Initial dietary rations used in farm 1 and the corrected ration ( ) in relation to the herd's potential

	Ingredients	kg/cow.day (DM)	Net energy (Mcal)	Metabolizable protein (g)	Rumen degradable protein (g)
Fodder	Green alfalfa	4.2 (6.8)	5.59 (9.06)	292 (473)	367 (594)
	Maize silage	2.3 (2.3)	2.37 (2.37)	145 (145)	127 (127)
	Wheat straw	1.2 (1.8)	1.04 (1.56)	62 (93)	32 (48)
Concentrates	Maize grain	3.5 (3.5)	7.41 (7.41)	420 (420)	296 (296)
	Beet pulp	1.6 (3.2)	2.93 (5.86)	167 (334)	125 (250)
Total nutrients' supply	-	-	19.34	1,086	947
Maintenance	-	-	9.00	420	420
True milk yield (kg/cow.day)	-	-	13.2	13.9	11.0
Potential milk yield (kg/cow.day)	-	-	22.6	22.8	18.6

Supplementation of the initial ration was thus proposed to improve the herds' average milk production. This consisted mainly in adding sources of degradable energy in the diet. Table 3 also shows the proposed ration with a balanced supply of nutrients to match the herd's potential production. In one month, supplementation increased the volume of milk per lactating cow in the herd from 11 to 19 kg, by supplying the adequate amounts of net energy and MP.

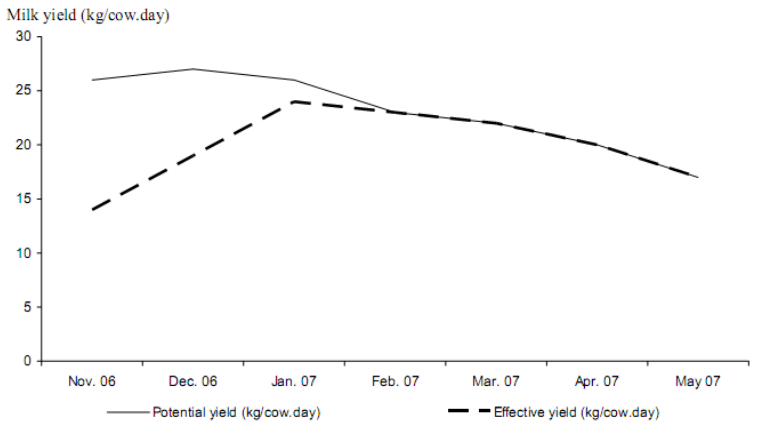
The concept of balancing the supply of nutrients in the dietary rations with changes in the herds' potential requirements was adopted in the five herds throughout the study period. The effects of a continuous correction of the dietary rations are shown in Figure 1. In farm 1, which adopted the strategy straight away, an effective milk yield equal to the potential milk capacity was reached just after three months.The farmer was able to judge the effects of the method on the profitability of the dairy herd (Table 4).The support process was also successful in another farm (farm 4)with crossbred cows, but it took more than five months to reach its potential milk yield (Figure 2). This result highlights the quicker response of purebred Holstein cows to improved dietary rations than that of crossbred cows. This can be explained by the better milking ability of the Holstein breed which allows itconverting nutrients into milk more efficiently than other breeds (Delaby *et al*., 2009). However, in farm 5, the intervention did not work, as the farmer was not convinced and therefore milk production cost remained higher than farm gate milk price (Table 4). Increasing milk production to reach the potential allowed the milk production costs to be reduced below the farm gate milk price (0.35 US $/kg), making this activity profitable for four farms out of five.

Table 4. Changes in the daily gross margin per lactating cow and milk production cost during the intervention

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Initial gross margin (US $/cow.day)	- 0.5	- 2.9	- 1.9	- 0.4	- 1.3
Average gross margin* (US $/cow.day)	0.4 / 1.0	0.3 / 0.9	0.5 / 3.9	0.05 / 0.1	- 0.8 / - 1.3
Initial milk production cost (US $/kg)	0.4	0.4	0.5	0.4	0.6
Average milk production cost (US $/kg)	0.2	0.3	0.4	0.3	0.5

* Variation values during the support process (minimum/maximum)

(a)



(b)

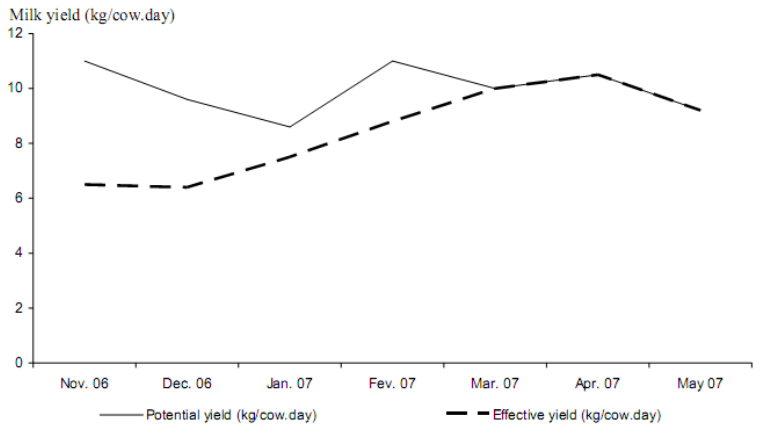


Figure 2. Effects of the support programme on the average milk yield for Holstein (Farm 1) and crossbred cows (Farm 4). a) herd of 17 pure Holstein cows (Farm 1). b) herd of 6 crossbred (Holstein x local) cows (Farm 4).

All together, the results of such an intervention in smallholder facilities demonstrate the possibility to promote cows' milk yield by a close monitoring of their dietary rations. This requires a relationship of confidence and proximity between researchers and farmers who need to be convinced by significant results.

MODELING MILK QUALITY PARAMETERS IN RELATION TO REARING PRACTICES

In order to assess milk quality and its variability among small scale farms involved in a fragmented dairy chain throughout a whole year period, a sample of 23 cattle units was chosen. They were monitored on a monthly basis from December 2005 to November 2006. Farm sampling expressed diversity of milk production strategies: three specialized dairy farms, a vast majority (n = 14) of dual purpose herds (group 2), and six farms in which cattle breeding had a secondary economic role (group 3). 151 milked cows were recorded in the study sample. Herds' size ranged from 1 to 22 cows per farm, while forage area varied from 0.5 to 18 ha with an average of 32% of the total area, ranging from 6 to 100% of arable land. A milk sample was then immediately pooled from bulk recipient milk.Raw milk pH and temperature were directly measured at the farm, just after the morning milking.

Milk samples were kept in sterilized glass bottles and transferred in a cooler to the analytical laboratory. All quality analyses were realized in less than 24h. Milk fat, protein and solids not fat contents were determined by an infrared spectrophotometer (Milksocan Foss FT 2®, Foss Electric, Hillerod, Denmark). Aerobic Plate Count (APC) analyzes were undertaken following the standard International Dairy Federation (IDF) protocol (IDF, 1987). Coliforms' contents were also determined by IDF methods (IDF, 1974). All microorganisms' counts (APC and Coliforms) were expressed in colony forming units (cfu) per ml.

Dietary rationswere recorded and energy supply from concentrates was determined in each herd. For that purpose, the focus was on the quantity of concentrates distributed to milked cows during the day of milk sampling. Their net energy supply was determined from feed tables (Jarrige,1988).

Two explanatory variables of the milk hygienic quality parameters were also recorded. On the one hand, hygiene during milking was controlled by monitoring hand, udder and teat washing, elimination of foremilk and type of recipient used. Cows' dirtiness score was calculated according to the method described by Faye and Barnouin (1985), which relies on the score of 5 body areas between udder and forelimb. On the other hand, the reproduction status of the entire herd was monitored. The lactation stage of each herd was determined monthly by the follow-up of calving rhythms (Equation *1*).

Results showed that average cows' milk chemical parameters (38 and 30 g/kg for fat and protein contents) were within international standards for that

product, as alfalfa was an adapted roughage to sustain good quality production (Walker et al., 2004). The annual follow-up of a broad diversity of cattle farming units also allowed identifying the rearing practices which significantly affect milk quality parameters. Highly significant models were established which link fat and protein contents to on-farm breeding practices. These models were as follows:

$$Fat = 37.7 + 0.36 \times Lactation_Stage - 2.34 \times Energy + Genotype_{FAT}$$

with:

$Lactation_Stage$: Average lactation stage in the herd (in months)

$Energy$: Net energy provided by concentrates per kg of milk (MCal)

$Genotype_{FAT}$: Genotype effect estimated as such by the model (Figure 2):

Exclusive Holstein: -3.5 g/kg

Mixed (Holstein and crossbreds cows): -1.5 g/kg

Exclusive crossbred: -1.2 g/kg.

Local strain: 0 g/kg.

$$PRotein = 26.7 + Genotype_{PRO} + Interaction_{Gen \times Lactation} + Interaction_{Gen \times Yield}$$

with:

$Genotype_{PRO}$: Genotype effect estimated as such by the model:

Exclusive Holstein: -1.7 g/kg

Mixed (Holstein and crossbreds cows): -1.2 g/kg

Exclusive crossbred: -1.0 g/kg.

Local strain: 0 g/kg.

$Interaction_{Gen \times Lactation}$: Interaction between genotype and lactation stage (significant only for exclusive Holstein herds, +0.84 g/kg).

$Interaction_{Gen \times Yield}$: Interaction between genotype and average milk yield per cow (significant only for exclusive Holstein herds,+ 0.34 g/kg)

These models show that the herd genetic structure affects the chemical quality (fat content) directly or in combination with other components (protein content) impacting milk yield. This component is stable in the short term, as farmers need time or capital to change it either by internal selection or by investing in new genetic material such as Holstein cows. Reproduction practices have an effect on the lactation stage and also on the chemical quality, but they are hardly controlled by small scale farmers. Indeed, insemination

failures disturb significantly the milk production dynamics in such small herds.

Finally, feeding practices affect milk fat content based on concentrates supply, and the protein content based on the total diet which controls milk productivity per cow for a given genetic type. These practices depend mainly on other components of the farm management, such as cash-flow to buy concentrates or irrigation management for forages. All together, results confirm that the highest values of fat or protein contents were related to the lowest daily milk yields per cow. They were observed in herds in which cows were rather fed unbalanced dietary rations or were close to drying up, or were of local breeds which have a limited lactation potential. These effects were attributed to the milk dilution process (Gandini et al., 2007).

The average milk hygienic quality was poor, as its indicators (APC: 7.4 x 10^5 cfu/ml and Coliforms: 5.1 x 10^4 cfu/ml) were more than 100-fold higher than international standards(Bramley and Mc Kinnon, 1990). These indicators were affected significantly by on-farm milking and hygienic conditions. They vary between farms, since they are very stable throughout the year in a given farm. Finally, the period of the year affects primarily the hygienic quality components, even for the farms with clean milking practices(Table 5).

Table 5. Factors of variation of milk hygienic quality parameters[1]

	Log Aerobic Plate Count (cfu/ml)	Log Coliforms (cfu/ml)
Period of milk production		
Cool and wet months	> 6.00[a]	> 4.51[a]
Hot months	< 5.51[b]	< 2.53[b]
Hand washing		
Yes	5.73[a]	4.25[a]
No	5.84[b]	4.83[b]
Type of milking		
Hand	5.89	4.68
Automatic	5.88	4.71

[1] average over the whole farm sample.

a, b: variable means within each column with different superscripts are significantly different (P < 001).

The close relationship between cattle breeding and milking practices on the one hand and milk quality components on the other hand, suggest

developing methodologies based on a list of production specifications. Such an indirect system of assessing milk quality could be proposed and tested in the Tadla irrigated scheme to reward farmers' efforts in improving quality. Some specification parameters are easily recordable, such as herd genetic structure, month of delivery or technical assets such as aluminium cans. Other ones are homogeneous enough within farms to require yearly control, such as milking practices. Feeding practices and lactation stages remain more difficult to monitor as they may frequently change within a farm according to its resources' availability or the hazards met in the course of production and reproduction processes. They will require frequent proximate controls, which may hinder the overall cost of the whole system.

CONCLUSION

This paper presents a reflection on the possibilities to increase the value in dairy chains where numerous small scale cattle farms are active. To achieve that goal, a preliminary diagnosis was conducted in a sample of dual purpose irrigated farms and it revealed that improper agricultural practices led to important drawbacks, as the average milk yield and profitability per cow were limited. This also implied poor water productivity through cattle farming, which could hinder the sustainability of that activity in the future, in a context of increasing water stress. Then, an on-farm intervention was conceived to enhance cows' milk yield through a continuous evaluation and correction of their dietary rations. The results were satisfactory, as the intervention allowed to match the effective milk yield to cows' potential productivity, whatever their genetic merit (rather purebred Holstein or crosses with local strains). That required however a proximate follow-up of farms. A second intervention was planned to assess the variability of milk quality indicators and its relationship to rearing practices. That allowed modeling milk quality indicators and suggests that in a chain with a fragmented offer, a monitoring of rearing and hygiene practices may be sufficient enough to remunerate milk batches delivered at their effective quality. All together, these results imply that many efforts are still needed to characterize dairy production systems and their dynamics in chains with a fragmented offer. When it comes to intervening in such farms, this requires adapted tools and sound investments. At a time, where State services are progressively disengaging from agriculture extension activities, interventions in a large population of small scale farms remain difficult to plan. Even though our results show encouraging evidence that

small scale farms' performances may be easily enhanced by proper advice, the generalization of such interventions in a wider population (for example in a whole irrigated scheme or nationwide) would necessitate important financial means and a sound logistical organization. The ongoing negotiations between Morocco and the European Union for a free trade agreement, which include agricultural goods, may constitute an opportunity for local operators in the dairy chain to consider ways to upgrade their performances. That would allow them staying resilient in front of imported dairy goods from Europe. If so, options of on-farm interventions, similar to those adopted in this study may be convenient. However, questions remain on their feasibility and on the actors which may handle them. Therefore, consequent efforts must be made by all the stakeholders in the Moroccan dairy chain for better governance, which should include interventions to support dairy farmers' correct feeding of their herds and also indirect monitoring of milk quality. This would require arrangements between all the stakeholders of the supply chain to cover the costs of such services (which would benefit them all), based on an increasing amount of raw milk of better quality delivered by farmers to processors through the milk collection co-operatives.

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Chapter 5

GENETIC PARAMETERS FOR CLINICAL MASTITIS AND SOMATIC CELL COUNT FOR HOLSTEIN COWS MANAGED UNDER MEDITERRANEAN CLIMATIC CONDITIONS IN TUNISIA

Naceur M'hamdi[1*], Rafik Aloulou[1], Mahdi Bouallegue[2], Satinder Kaur Brar[3] and Mohamed Ben Hamouda[4]

[1]Département des Sciences Animales, Institut Supérieur Agronomique de Chott-Mariem, BP47, 4042. Sousse, Tunisie

[2] Faculté des Sciences Mathématiques Physiques et Naturelle de Tunis, Campus Universitaire 2092 Tunis, Tunisie

[3] Institut National de la Recherche Scientifique, Québec, Canada

[4]Institution de La Recherche et de l'Enseignement Supérieur Agricole, (IRESA), Ministère de l'Agriculture et des Ressources Hydrauliques, Tunisie

[*] Corresponding address: Département des Sciences Animales, Institut Supérieur Agronomique de Chott-Mariem, BP47, 4042. Sousse, Tunisie. Tel: (216) 73 348 544- Fax: (216) 73 327 591. Email: naceur_mhamdi@yahoo.fr.

ABSTRACT

The goal of this chapter was to estimate genetic parameters for clinical mastitis (CM) and somatic cell count (SCC) in the first three lactations of Tunisian Holstein cows in order to define how to include this trait as a selection criterion. Mastitis, an inflammatory disease of the mammary gland generally caused by intramammary infections, is the most frequently occurring disease in Tunisian dairy farms. Hence, reduced milk yield, milk quality, and lactation persistency as well as early culling contribute to the economic losses associated with this disease. Mastitis problems were assumed to decrease profitability of dairy cows through milk price, treatment and involuntary culling costs. Somatic cell count (SCC) and clinical mastitis (CM) were analyzed with mixed linear model using data from the first three lactations of 7120 Tunisian Holstein cows having their first calving between 1996 and 2003. Somatic cell counts were log-transformed to somatic cell scores (SCS). The model included fixed effects of year-month and age at calving, and random effects of herd-year at calving and sire. SCC in milk increased as parity increased. The heritability estimates range from 0.009 to 0.12 and from 0.01 to 0.03 for SCC and CM, respectively. The higher genetic correlation between SCC and CM (average 0.65) imply that SCC is an appropriate indicator of the infectious status of the mammary gland. All genetic correlations between CM and SCS were positive, implying that genetic selection on lower SCC will reduce CM-incidence.

Keywords: Cattle, heritability, mastitis somatic cells score, variance

1. INTRODUCTION

Holstein cows were considered the most dairy cows in world. Holstein becomes more prevalent day by day, as is generally the case in most of the countries of the world, due to their good adaptation to different climates under good management [1; 2] and its high milk and meat production, this breed is the mainly demanded one among culture cows in Tunisia [3]. However, Akbulut *et al.* [4] and Atasever and Erdem [5] clearly indicated that milk production levels of this breed is highly lower than those raised in the US and EU countries. Mastitis resistance is one of the most important of these traits, and reducing incidence of this disease through genetic selection is of great interest both for economical and animal welfare reasons [6]. Mastitis is an

inflammatory disease of the mammary gland. It is the most frequently occurring disease in dairy cows, and economic losses associated with this disease are attributed to reduced milk yield and quality, reduced lactation persistency, and early culling [7; 8]. In recent years, selection for improved resistance to mastitis has become increasingly important in dairy cattle breeding programs [9]. Economic losses from mastitis are considerable and result from reduced milk yield, discarded milk, and reduction in milk price because of high SCC, veterinary fees and treatment costs, increased labor and culling rate. Aspects regarding food safety and animal welfare, such as the increased use of antibiotics, are also strong arguments for reducing the frequency of mastitis. According to Heringstad *et al.* [10], Hansen *et al.* [11] and Carlén *et al.* [12], milk production is unfavorably genetically correlated with clinical mastitis (CM) and this emphasizes the need to include mastitis resistance in the breeding goal. Somatic cell count (SCC) in milk is one of the best indicators of udder health status.It is relatively easy and inexpensive to collect in combination with routinely milk recording. High SCC reduces the quality of milk and dairy products [13].Management and breeding decisions aim to reduce SCC, as a way to decrease the incidence of mastitis [14]. For SCS, a large number of estimates of heritabilities and variance components are reported in literature [15]. heritabilities estimates ranged from 0.05 to 0.19 [16; 17; 18]. Most of these estimates were based on the average SCS during the lactation, calculated from monthly records or from pre-corrected monthly records. Fewer estimates were computed using a test-day model [16; 19].

2. MATERIAL AND METHODS

2.1. Data

Data were extracted from the Tunisian Livestock and Pasture Office (OEP) and were edited to include records from the first three lactations of Tunisian Holstein cows having their first calving between 1996 and 2003. Data contained multiple somatic cell count (SCC) measurements made during the lactation months for each cow. Although information on lactation number was recorded, a general restriction of age at calving was constructed to exclude cows with suspected wrong lactation number. The defined minimum and maximum ages for the first, second, and third calving were 20 to 38, 32 to 52, and 43 to 66 months, respectively. If age at calving at a particular lactation was below or above the allowed period, records for that lactation were not

used in analyses. Data editing was done by excluding cows having more than three parities and age at first calving less than 20 months. Only test-day SCC recordings between 1 and 335 DIM and only observations on CM between 1 and 335 DIM were included. Ten days in milk (DIM) classes were defined. The first class included test days between 5 and 30 DIM and all other classes were 30 days long. The structure of the analyzed data for lactations 1 to 3 is shown in Table 1.

Table 1. structure of the used data

Source	Lactation		
	1	2	3
Lactation, no.	6520	5470	3175
Sires, no.	112	98	79
Sires, no.	27.611	19.025	11.570

Pedigree information of the sires of cows in the data set was traced back as far as possible, resulting in a sire pedigree file with 139 bulls, including the sires with daughter records. Individual SCS values are averaged over all months of lactation and adjusted for effects of age, stage of lactation, and month of calving [20]. The adjusted average SCS for each lactation of each cow is used in the genetic analysis.

2.2. Statistical Analysis

Conventional statistical methods usually assume normally distributed data and homogeneity of variance. The value of SCC is therefore log-transformed to somatic cell score (SCS) by the following formula:

$$SCC = \log_2 (SCC/100) + 3 \text{ [21]}$$

SCC variation was evaluated by the general linear model (GLM) [22].To test the effect of non-genetic factors, a pre-analysis using GLM procedure was done. CM was scored as present (1) if a test-day SCC >150.000 cells ml^{-1} was registered; otherwise, it was scored as absent (0). The threshold of 150.000 cells ml^{-1} was chosen based on the lowest threshold used by NRS to highlight animals with high SCC [23].

The statistical analysis of longitudinal data was carried out using MTDFREML (Multitraits Derivative Free Restricted Maximum Likelihood) animal model [24]. The general linear mixed model is given by:

$$SCS_{ijklm} = \mu + H_i + NL_j + DIM_k + CM_l + (HDIM)_{ik} + (NLDIM)_{jk} + pe_{m+} a_m + e_{ijklm}$$

where: SCS_{ijklm} is test-day observation m on cow l, μ is the overall mean, H_i is the fixed effect of herd, NL_j is the fixed effect of number of lactation j, DIM_k is the fixed effect of stage of lactation k, CM_k is the fixed effect of calving month k, $(HDIM)_{ik}$ is the fixed effect of the interaction between herd i and lactation stage k, $(NLDIM)_{jk}$ is the fixed effect of the interaction between parity j and lactation stage k, pe_m is a random effect accounting for permanent environmental effect associated with all test-day records of cow l, a_m is the random additive genetic effect of cow l and e_{ijklm} is the random residual error term. To quantify the herd-by-sire interaction effect, the model was extended by including h*s random effect, were h is the herd and s is the sire of the animal respectively. Levels with only 1 record were deleted. To account for additive genetic relationships, a pedigree files was extracted from OEP. MTDFREML procedure was used to estimate additive and non-additive variances and co-variances for SCC and SCS.

A variance structure from the sire model in general terms is:

$$\sigma_p^2 = \sigma_s^2 + \sigma_e^2 \quad (1)$$

where σ^2_p is the total phenotypic variance, σ^2_s is the sire component of variance and σ^2_e composed of 3/4 the additive genetic variance along with the non-additive genetic variance and environmental variance. The cow model was used in a separate analysis of variance and the structure of variance component was:

$$\sigma_p^2 = \sigma_c^2 + \sigma_e^2 \quad (2)$$

$$\sigma_c^2 = \sigma_a^2 + \sigma_{Ep}^2 \quad (3)$$

where σ^2_p is the total phenotypic variance, σ^2_c is the cow component of variance, σ^2_a is the additive genetic variance, σ^2_{Ep} is the permanent environmental variance and σ^2_e is the residual variance. Heritability (h^2),

genetic (Rg) and phenotypic (Rp) correlation between the two traits was estimated following Lowry [25];

$$h^2 = \frac{4\sigma_s^2}{\sigma^2} \quad (4)$$

$$R_g = \frac{\sigma_{sisj}}{\sqrt{\sigma_{si}{}^2}\sqrt{\sigma_{sj}{}^2}^2} \quad (5)$$

$$R_p = \frac{\sigma_{pipj}}{\sqrt{\sigma_{pi}{}^2}\sqrt{\sigma_{pj}{}^2}^2} \quad (6)$$

where σ^2_{sisj} and σ^2_{pipj} are sire genetic and phenotypic covariance between i^{th} trait and the j^{th} trait, respectively and are estimated from the analysis of measurements of the two traits on the same animal. σ^2_{si}, σ^2_{sj} are sire genetic variance and σ^2_{pi}, σ^2_{pj} are phenotypic variances for both traits i and j, respectively.

RESULTS AND DISCUSSIONS

Descriptive Statistics

The proportion of lactations with presence of SCC >150.000 cells ml^{-1} was roughly 73 %. The average length of the longest streak of consecutive test-days with SCC above 150.000 cells ml^{-1} was slightly longer (1.71 test-days). Across parities, milk production of mastitic animals was 0.55 kg higher than the milk production of the healthy animals with average value of 21.06 and 20.5, respectively, and somatic cell count was highest for mastitic animals (427.3 vs 147.8).

The largest difference between the SCC of mastitic compared to healthy animals was seen in parity 3 with average value ranged from 513.9 and 156.5, respectively (Table 2). In the considered data, the age at calving did not have a significant effect in the pre-analysis using GLM procedure and therefore it was not included in the model of analysis. The lack of significance was may be due to the fact that only first parity cows were considered.

Table 2. Average daily milk production (kg) and somatic cell count (SCC) (1000 cells/ml) for healthy (0) and mastitic (1) animals

Parity	CM	Milk	SCC
	0	20.5	147.8
All	1	21.06	427.3
	0	19.8	133.7
1st parity	1	20.35	361.6
	0	21.3	149.7
2nd parity	1	21.86	447.6
	0	21.8	156.5
3rd parity	1	22.37	513.9

Analysis of Variance

The herd had a significant effect on SCC ($P< 0.01$). The proportion of total variance explained by sire by herd effect ranged from 0 .004 to 0 .015 (Table 3). The inclusion of the sire by herd effect in the model only slightly decreased the estimates of additive and permanent variance. The results of likelihood ratio test confirmed that the likelihood of a model including a sire by herd effect did not significantly differ from the likelihood of a model ignoring such an effect. Statically significant differences among herds found in this study for SCC levels were agreed with some results found by [26; 27; 28]. The SCC levels for herds in this study were generally lower than results found by [26; 27] but, similar to Koç [28; 29] results under Mediterranean climatic conditions and Fernandes *et al*. [30] in Brazilian conditions rearing.

Table 3. Heritabilities (h2) and genetic variances (σa2) with standard errors in parentheses, of clinical mastitis and several SCC traits

Trait	h^2	σ_a^2
CM	0.025 (0.01)	0.002 (0.001)
SCS100	0.08 (0.01)	1.713 (262.2)
SCS150	0.09 (0.02)	1.740 (256.7)
SCS200	0.11 (0.01)	1.816 (257.2)
SCS250	0.12 (0.02)	1.731 (241.8)
SCS300	0.13 (0.02)	1.785 (240.05)
SCS350	0.12 (0.02)	1.781 (236.9)
SCS400	0.13 (0.02)	1.750 (232.8)
SCS151- 400	0.13 (0.02)	1.844 (247.8)
SCS335	0.12 (0.01)	1.639 (220.1)

The differences between the herds SCC means were resulted from different managerial practices, barn conditions, milking management and milk

hygiene. However, the mean for Tunisian Holstein population (147.800 and 427.300 cells ml^{-1} for healthy and mastitic animals, respectively) was higher than in European countries [31; 13]. We noticed a difference among primiparous and multiparus; indeed, multiparus have, on the whole, higher SCC. Figure 1 illustrates the relative incidence of CM within the first three lactations of Tunisian Holstein-Friesian cows. We pointed out that parity affects significantly ($P< 0.001$) incidence of mastitis. However, it seems that the incidence of CM increases with increasing parity and is highest in early lactation.

Göncü, Özkütük [26] and De Haas [32] were reported higher SCC levels for later parities. Increasing parity was reported a risk factor for increasing prevalence of clinical mastitis incidences [33]. In agreement with the present study, Hagnestam *et al.* [34] noticed that incidence of CM is highest in first-parity cows (figure 1).

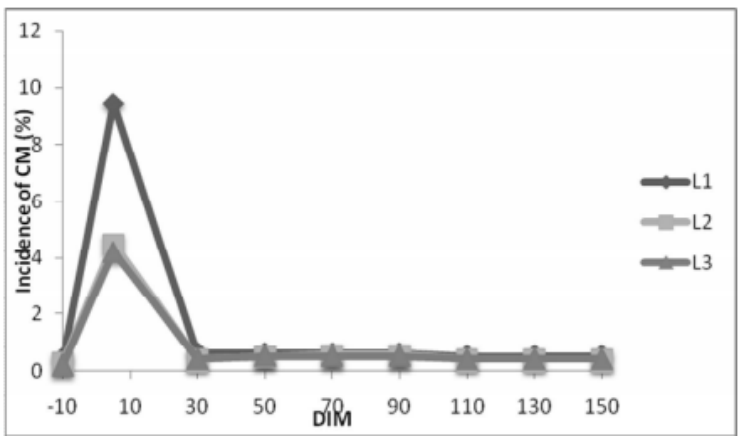


Figure 1. Relative incidence of the total number of CM cases within cases 150 days of the first three lactations (L1, L2, and L3).

Lactation stage affects significantly SCC. The mean SCC of the milk from animals whose lactation stage was under 150 days during the first period was significantly lower ($P< 0.001$) than that of cows whose lactation stage was above 150 days. As seen in Figure 2, SCC means for lactation months increased gradually from the second month of lactation to the end of lactation. SCC in the first month of lactation was 607.295 cells ml^{-1} and dropped to 387.525 in the second month of lactation. The lowest SCC was in the second and third lactation months. SCC in these months was below 400.000 cells ml^{-1} and started to increase in the fourth month, continuing to increase until the end

of lactation. From month 4 to 8, SCC was between 400.000 and 500.000 cells ml^{-1}. Then, it increased to over 500.000 cells ml^{-1} in the last two months of the lactation. The results found in this study are in agreement with those of Koç [35; 27] in Mediterranean climatic conditions. SCC during the first month of lactation was higher than in other months. This result agrees with the results of Sederevicius *et al.*[36] (figure 2).

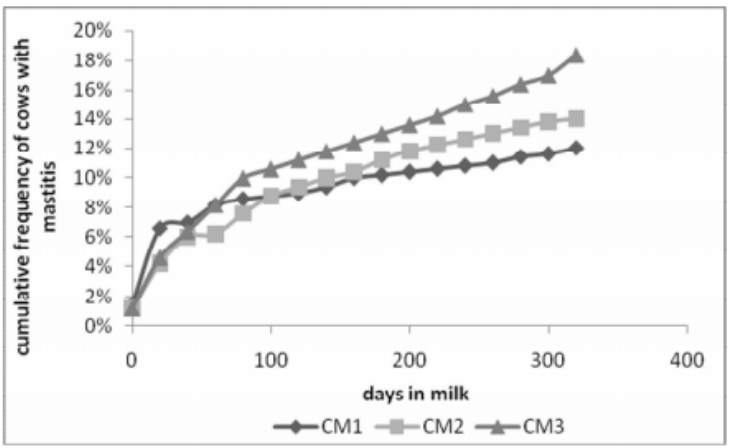


Figure 2. The cumulative frequency of animals with at least one case of clinical mastitis during parity 1, 2, or 3 (CM1, CM2, and CM3, respectively) per day in milk.

Univariate Analyses

Heritabilities for lactation-average SCS varied from 0.08 to 0.13 and increased with longer lengths of lactation. Heritability of SCS335 was 0.12. Generally, the heritabilities of SCC traits were lower than estimates for lactation-average SCS, but greater than estimates for CM. The heritability of CM was 0.02 (Table 3).

Heritability estimates of CM were low but in the range of 0.01– 0.06 reported by Heringstad *et al.* [1], Carlén *et al.* [12] and Bloemhof *et al. [37]*. Overall, the heritabilities estimated in the current study tend to be higher than the heritabilities estimated by De Haas *et al.* [39]. The heritabilities for CM in parities1, 2, and 3 were around 3% (Table 3). Heritability estimates of parity-specific CM were low but in the range of 0.01– 0.06 reported in previous studies using linear models [37; 10; 12]. However, CM is an all-or-none trait and heritability estimates on the linear scale are therefore frequency

dependent. Heritability estimates of different studies are therefore not easily comparable (Heringstad *et al. [10]*.

Bivariate Analyses

The estimated genetic correlations between CM and SCS were 0.68 ± 0.06. The genetic correlation between SCC and CM is moderate to high (often around 0.6- 0.8), suggesting that genes predisposing cows to a low SCC also result in a lower rate of CM (10; 40; 41; 42]. Genetic correlations with lactation-average SCS over longer periods were (> 0.66) for CM. The estimated genetic correlations between CM and SCS335 were 0.64 ± 0.08. The genetic correlations between SCS335 and the alternative SCC traits were high (Table 3), ranging from 0.74 to 0.99. Near-unity genetic correlations were estimated between SCS335 and SCS300, SCS350, and SCS400.

Table 4. Genetic correlations between clinical mastitis in the three first parities and somatic cell scores from 5 to 335, from 5 to 150 and from 151 to 335 days, respectively, with standard errors in parentheses

Trait	CM1	CM2	CM3
SCS5-335	0.64 (0.12)	0.79 (0.10)	0.79 (0.10)
SCS5-150	0.65 (0.11)	0.86 (0.08)	0.88 (0.09
SCS151-335	0. 50 (0.15)	0.59(0. 14)	0.61 (0.14)

Table 5. Estimated parameters for clinical mastitis in the three first parities

Trait	CM1	CM2	CM3
CM1	0.03 (0.01)	0.88 (0.13)	0.63 (0.22)
CM2	0.06 (0.01)	0.03 (0.01)	0.91 (0.12)
CM3	0.05 (0.02)	0.12 (0.01)	0.04 (0.01)

Heritabilities on diagonal, phenotypic correlations below diagonal and genetic correlations above diagonal, with standard errors in parentheses.

The genetic correlations between CM and lactation average SCS were 0.8 (0.1) in parity 2 and 3 (Table 5), but somewhat lower in first parity (0.74 ± 0.11). The genetic correlations between CM in consecutive lactations (i.e.1 and 2, and 2 and 3) were higher than the genetic correlation between CM in

non-adjacent lactations (1 and 3). This is in agreement with the results from previous studies where the highest genetic correlations were estimated between CM in second and third parity and lowest between CM in first and third parity [37; 12].This implies that CM in consecutive lactations is, genetically, more the same trait, than CM in non-adjacent lactations (Table 4).

Rupp *et al.*[43] also showed that udder health in first and second parity was genetically correlated. They concluded that animals with the lowest mean SCC in the first lactation had the lowest risk for CM in the second lactation. The lower genetic correlation estimated between first and third lactation (0.63 ± 0.22) could be due to selection. Animals that suffered from mastitis might be removed from the herd and were not fulfilling three lactations. Parity specific CM and lactation-average SCS over three Parities showed a moderate favorable genetic correlation in first parity (0.64), and strong favorable genetic correlations in later parities (0.79). The favorable geneticcorrelations imply that selection for lower lactation-average SCS will decrease the incidence of CM. Similar parity-specific genetic correlations were estimated by Carlén *et al.* [12], who also observed the highest geneticcorrelation between CM in parity 3and lactation-average SCS. In general, the estimated genetic correlations are in line with those reported in literature [15; 12]. Lower genetic correlations were estimated between CM and average SCS in the latter lactation, than Between CM and average SCS in the first lactation. Standard errors were quite large, and correlations should be interpreted with caution. Even so, the estimated genetic correlations implied that selection for Lower SCS, especially during early lactation, decreases the Incidence of CM. Emanuelson et al. [44] reported a similar trend in the estimated genetic correlations in three separate Swedish data sets. The genetic correlations estimated in our study between CM and average SCS in the first lactation were stronger. This implied that a real difference exists in the Genetic resistance to CM between different lactations, which is consistent with earlier results [44; 39]

CONCLUSION

SCC has several desirable attributes as an indicator trait for CM, and its use for this purpose is therefore widespread. It is necessary to include functional traits, such as mastitis resistance, in the breeding goal if we are to prevent deterioration of the kind consequential upon selection for production only, as well as to meet the economic, animal welfare and ethical concerns of

farmers, consumers and the society. Genetic selection can be performed by direct or indirect selection, or by a combination of both approaches. The choice of approach depends on the breeding goal, the availability and accuracy of records, the population structure and the genetic parameters of goal and indicator traits. The higher heritability for lactation average SCC than for CM, and its high genetic correlation to CM, makes it a suitable trait to use for indirect selection to improve mastitis resistance.

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Chapter 6

INCIDENCE OF HYPOCALCEMIA AND ITS CA HOMEOSTASIS MECHANISM IN PERIPARTURIENT COWSFROM THREE INTENSIVE DAIRY FARMSOF HEILONGJIANG

C. Xia, H. Y. Zhang, C. Xu, L. Wu, J. S. Zheng, S. B. Sang and L. J. Yang
Animal Veterinary key Lab, Department of Veterinary Medicine, College of Animal Science and Technology, Heilongjiang Bayi Agricultural University, Daqing, 163319 China

ABSTRACT

The objective of this study was to understand status of hypocalcaemia and Ca homeostasis of the local dairy cows during transition period. Sixty multiparous Holstein cows from three intensive dairy farms (I, II, and III, 20 cows per farm) of Heilongjiang province in China were randomly assigned to this experiment in transition period. Their dietary cation-anion difference (DCAD) was in turn 91 meqkg-1 of DM, 152 meqkg-1 of DM, and 85 meqkg-1 of DM, respectively. Concentrations of plasma Ca, hydroxyproline (HYP) 1,25-dihydroxyvitamin D (DHVD), and parathyroid hormone (PTH) were determined at d 21, 14, and7before expected calving, at calving, and at d 7, 14, and 21 after calving. In three farms, the incidence of hypocalcaemia increased near time of calving, reached to the highest at

calving (>75%) and then decreased after calving, and plasma Ca was just opposite to it. Compared to other farms, cows in farm II fed a greater positive DCAD had a higher incidence of hypocalcaemia, a lower concentration of plasma Ca and HYP at calving ($P < 0.05$), which indicates that high DCAD inhibited bone Ca mobilization. In addition, cows fed a high positive DCAD in farm II had a slight increase of plasma DHVD concentration ($P > 0.05$)and a higher concentration of plasma PTH at calving ($P < 0.05$), which implicates target tissues were refractory to PTH stimulation. These data demonstrated that hypocalcaemia is very popular during transition period in local three dairy farms. The high DCAD is a major risk factor for hypocalcaemia, which may reduces ability of the cow to maintain Ca homeostasis.

Keywords: Dairy cows, Hypocalcaemia, DCAD, Ca Homeostasis

ABBREVIATIONS

DCAD,
dietary cation-anion difference;
DM, dry matter;
DHVD,
1,25-dihydroxyvitamin D
HYP,
hydroxyproline;
PTH,
parathyroid hormone

Milk fever or hypocalcemiais a metabolic disorder in which Ca homeostatic mechanisms fail to maintain normal plasma Ca concentrations at the beginning of lactation (Goffand Horst1997). At the onset of lactation, Cahomeostatic mechanisms have to react to a tremendous increase in demand for Ca. Mobilization of Ca from bone and increased absorption from the gastrointestinal tract are required to reestablish homeostasis. The physiological control of calcium metabolism is normally under regulation of systemichormones, especially parathyroidhormone (PTH),calciton in (CT), and 1,25-dihydroxy vitamin D (DHVD) (Jorgensen 1974; Goff 2008). The bone metabolism is possible monitored by different proteins or enzymes released during bone formation or resorption. Hydroxypmline (HYP) is an

amino acid unique to collagen. Hydroxyproline content of plasma or urine has been effectively utilized as an indicator of bone collagen resorption activity. The parameters connected with resorption in the periparturient period have been considered (Liesegang et al. 2000) to understand and describe the mechanisms behind the complex and delicate system known as calcium homeostasis.

Parathyroid hormone has three main functions in cows: to mobilize Ca from bone, to promote absorption of Ca from the digestive tract by increasing DHVD concentration, and to stimulate the kidneys to excrete excess P while retaining Ca for reabsorption (Horst et al. 1997; Goff 2008). As blood Ca concentration decreases, PTH concentration increases. High concentrations of PTH promote the production of 1-α-hydroxylase in the kidney, the enzyme that converts 25-OH to DHVD. When blood Ca is within the normal range, PTH secretion decreases and DHVD is catabolized through a negative feedback mechanism. When DHVD or PTH bind to osteoblasts, the osteoblasts secrete compounds that activate osteoclasts to resorb bone mineral and return it to the blood(Goff 1991; Horst et al.1994).

Some studies (Block1984; Goff et al. 1991) have suggested that the response of kidney and bone to PTH is impaired in cows that develop milk fever and this responsiveness of the target tissues can be modified by the prepartum diet, especially high dietary Na and K, increase the susceptibility of cows to milk fever (Jonsson 1978; Goff 2006).

At present, little is known of status of hypocalcemia and its relationship with dietary cation-anion difference in intensive dairy farms from Heilongjiang province in China. Therefore, the purpose of the present study was 1) to investigate the incidence of cows` hypocalcemia and its regulative mechanism during transition period, and 2) to provide an evidence for relationship of high DCAD with hypocalcemia of the periparturient cows in the three intensive dairy farms from Heilongjiang province in China.

MATERIALS AND METHODS

Animals

All animals in this study were treated according to International Guiding Principles for Biomedical Research Involving Animals. Sixty dairy Holste in cows from three intensive dairy farms (I, II, and III, 20 cows per farm) in

China were selected for this experiment. The dairy cows were≥3th lactation. Farm I that locates at Honggang suburban of Daqing city in the western of Heilongjiang province, had 600 lactating cows, which were housed in scatter pens bedded with sawdust, fed a total mixed ration (TMR), which contained 20.6% corn grain, 6.47% concentrate (36% protein), 4.68% soybean cake, 1.95% rice cake, 2.11% greasy bran, 0.30% sodium bicarbonate, 0.68% calcium phosphate dibasic, 0.51% additive premix, 0.30% sodium chloride, 52.00% corn silage, 10.4% hay. Farm II that locate at Peide town in the eastern of Heilongjiang province, had 1500 lactating cows, which were housed in scatter pens bedded with sawdust, fed a TMR, which contained 21.4% corn grain, 3.20% concentrate (40% protein), 6.44% soybean cake, 0.48% fatty powder, 0.33% sodium bicarbonate, 0.15% calcium phosphate dibasic, 0.48% additive premix, 0.20% sodium chloride, 47.32% corn silage, 15% hay, 5% alfalfa. Farm III that locate at Mishan city in the eastern of Heilongjiang province, had 800 lactating cows, which were housed in individual fastening pens bedded with wood, fed a TMR, which contained 22.6% corn grain, 4.02 % concentrate (38% protein), 4.66% soybean cake, 3.10% wheat bran, 0.32% calcium phosphate dibasic, 0.45% additive premix, 0.15% sodium chloride, 52.10% corn silage, 12.6% alkali grass. These animals were fed the different diets according to the requirements and weight of dairy cows in transition period (Table1).The DCAD of three farms in transition period was calculated using the formula DCAD ($meqkg^{-1}$of DM)= [(Na + K) – (Cl + S)].All animals had free access to water. The diet was offered the rice daily in equal meals.

Sample Collection and Treatment

Blood samples were collected on d 21, 14, and 7 before parturition, on the day of parturition (0 d), and then at d 7, 14, and 21 after parturition. Blood samples were collected from the jugular vein into tubes with heparin at 6:00in the morning. Blood was centrifuged (1,500g, 10min) within 20min after collection. Plasma samples were stored at -80ºC until the analysis.

Plasma Analysis

Plasma Ca and HYP were determined by colorimetry with a semi-Auto analyzer (ECOM-F 6124, Germany), using commercial kits (Chang Chun Hui Li Bioengineering Lt Company). Plasma concentration of PTH was

measured at Nucleo-Radiology Department of Harbin Medical University in China using a commercially available radio immunoassay (No. of the kit 200803-2 from Atomic High Technology Limited Company, Beijing, China). Plasma concentrations of DHVD were measured at instution using a high performance liquid chromatography(HPLC)at Institute of Product Quality Monitoring in Jilin, China.

Hypocalcaemia and Milk Fever

A cow was considered to have milk fever if she stayed recumbent with a plasma Ca concentration < 1.40mmolL^{-1}. A cow was considered as subclinical hypocalcaemia if plasma Ca concentration was <1.90 mmolL^{-1} at any time during the experiment. A cow was in negative Ca balance if plasma Ca concentration fell to < 2.20 mmolL^{-1} at any time during the experiment (Goff and Horst 1997; Larsen, et al. 2001).

Statistical Analysis

Effects of treatments on the incidence of milk fever and hypocalcemia were assessed by chi-square analysis. A multivariate ANOVA with repeated measures (MA-NOVA, test within subjects) and a trend analysis (TREND, linear, quadratic, and cubic) were performed by the use of statistical software (Systat version 11.0, SPSS Inc., Chicago, IL). The differences were considered statistically significant if the P-value was < 0.05. All data were presented as means (± SD).

RESULTS

Content of dietary Na in farm II was relatively higher than that in other farms, of dietary Cl and S in farm II was relatively lower, and DCAD in farm II (152 meqkg-1) was higher than that in farms I (91 meqkg-1 of DM) and III (85 meqkg-1 of DM)(Table 1).

Table 1. Nutrients composition of the diets

Nutrients	Farm I	Farm II	Farm III
Dry matter (%)	72.25	75.62	74.34
Fat (%)	4.12	4.20	4.06
Crude fibre (%)	19.54	21.62	21.24
Crude protein (%)	10.50	11.50	10.40
NE_l [z] (Mcal kg^{-1})	1.60	1.64	1.56
Ca (%)	0.54	0.68	0.51
P (%)	0.31	0.48	0.32
Na (mg kg^{-1})	3625	4410	3841
K (mg kg^{-1})	6618	6539	6762
Cl (mg kg^{-1})	3025	2254	2755
S (mg kg^{-1})	2415	2315	2841
DCAD [y] (meq kg^{-1} of DM)	91	152	85

[z] NE_l,net energy for lactating.

[y] DCAD, dietary cation-anion difference, is calculated by a formula of [(Na + K) – (Cl+S)].

Table 2. Concentration of plasma Ca, HYP, PTH, and DHVD in three dairy farms during the trial

Parameters [z]	Farms	Prepartum [y]	Parturition [x]	Postpartum [w]
	I	2.36±0.30	1.95±0.29[b]	2.29±0.32[b]
Ca	II	2.27±0.16	1.80±0.23[a]	2.20±0.22[a]
	III	2.34±0.21	2.01±0.20[b]	2.28±0.19[b]
	I	2.47±0.18	2.62±0.21[a]	2.41±0.18[a]
HYP	II	2.36±0.21	2.32±0.20[b]	2.22±0.20[b]
	III	2.37±0.21	2.50±0.22[a]	2.36±0.15[a]
	I	155.77±18.50	190±25.30[a]	182±23.00
PTH	II	173±23.00	258±30.12[b]	220±25.60
	III	160±21.00	219±27.60[b]	194±22.00
	I	21.37±3.35	25.80±3.40	21.70±3.53
DHVD	II	23.70±4.23	27.51±4.10	24.30±4.70
	III	22.63±3.50	24.82±3.20	22.43±3.64
n		20	20	20

[z] Data are presented as mean±SD. Values superscripted by the different lower-case letters (P<0.05).

[y] Prepartum including d 14, 7 before calving.

[x] Parturition including the day at calving.

[w] Postpartum including d 7, 14 after calving.

Investigation of hypocalcemia (Figure 1) shows clearly that hypocalcaemia occurred at seven time point during the trial, and its incidence gradually increased before calving, reached to the highest at calving (above 75%), and gradually deceased to normal after calving. However, incidence of hypocalcemia in farm II was higher than other farms in transition period.

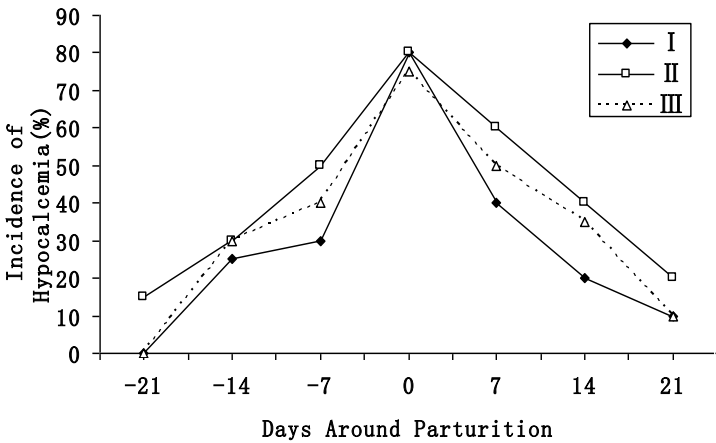


Figure 1. Incidence of hypocalcemia (plasma Ca <1.90 mmolL^{-1}.) in periparturient cows fed a diet with DCAD of 85 meqkg^{-1} of DM (I) , 152 meqkg^{-1} of DM (II), and 90 meqkg^{-1} of DM(III) diets prior to calving, respectively.

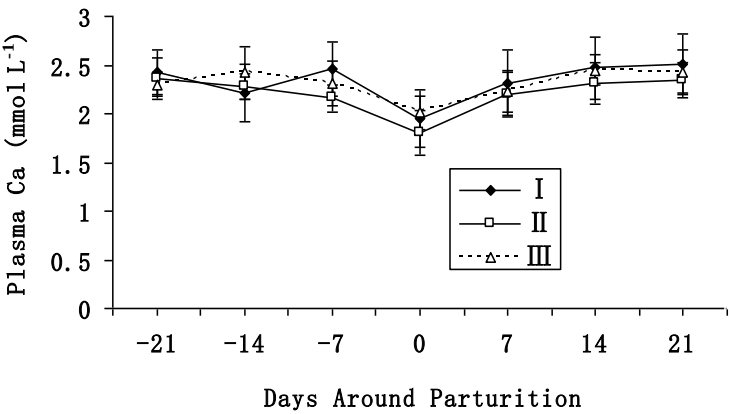


Figure 2. Profile of plasma Ca in periparturient cows fed a diet with DCAD of 85 meqkg^{-1} of DM (I) , 152 meqkg^{-1} of DM (II), and 90 meqkg^{-1} of DM(III) diets prior to calving, respectively. Bars represent standard deviations of the means.

Concentration of plasma Ca and HYP during the trial shows that both levels gradually decreased before calving, was the lowest at calving, and gradually increased after calving (Figure 2 and Figure 3). At calving, the decrease of plasma Ca and HYP for cows in farm II was significant compared to other farms ($P< 0.05$) (Table 2).

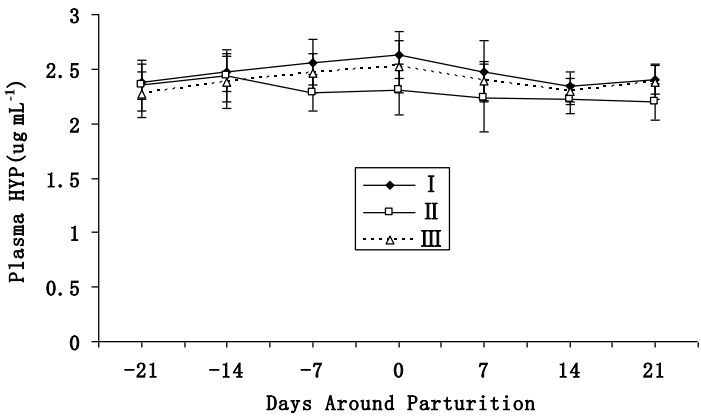


Figure3. Profile of plasma hydroxyproline (HYP) in per parturient cows fed a diet with DCAD of 85 meqkg^{-1} of DM (I) , 152 meqkg^{-1} of DM (II), and 90 meqkg^{-1} of DM(III) diets prior to calving, respectively. Bars represent standard deviations of the means.

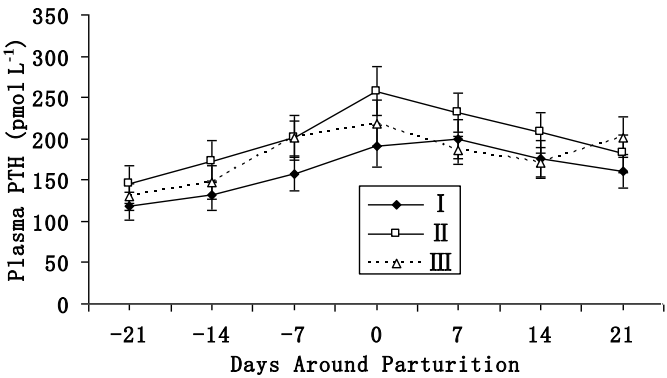


Figure 4. Profile of plasma parathyroid hormone (PTH) in periparturient cows fed a diet with DCAD of 85 meqkg^{-1} of DM (I) , 152 meqkg^{-1} of DM (II), and 90 meqkg^{-1} of DM(III) diets prior to calving, respectively. Bars represent standard deviations of the means.

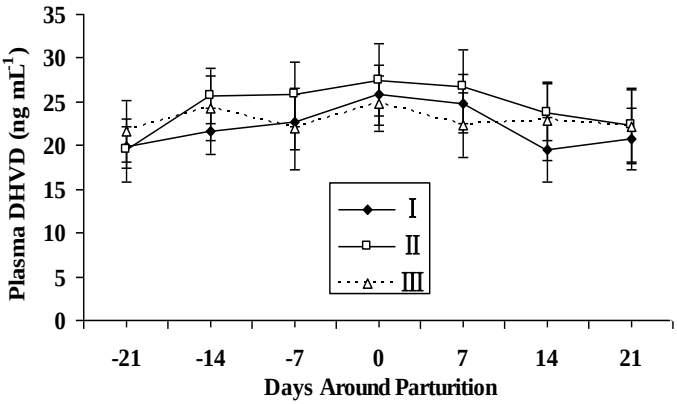


Figure 5. Profile of plasma 1,25-dihydroxyvitamin D (DHVD) in periparturient cows fed a diet with DCAD of 85 meqkg^{-1} of DM (I) , 152 meqkg^{-1} of DM (II), and 90 meqkg^{-1} of DM(III) diets prior to calving, respectively. Bars represent standard deviations of the means.

Concentration of plasma PTH and DHVD during the trial shows that both levels gradually increased before calving, was the highest at calving, and gradually decreased after calving (Figure 4 and Figure 5). At calving, the increase of only plasma PTH for cows in farm II was significant compared to other farms ($P< 0.05$) (Table 2).

DISCUSSION

Nutrient levels of the periparturient diets in three dairy farms is reported in Table 1. Based on analyzed nutrient concentration, the diets supplied available Ca and P to meet the cows' requirements and supplied energy and protein in approach of requirements (NRC, 2001). However, there was a marked difference in the dietary cation-anion difference (DCAD) among farm II (152 meqkg-1 of DM) and farm I (91 meqkg-1 of DM), farm III (85 meqkg-1 of DM), due to a difference of Na, Cl and S content in diets in three dairy farms, corresponding to a higher incidence of hypocalcemia in farm II compared to other farms during transition period (Figure 1).

Some evidences implicating the high cation-anion difference (CAD) of prepartum rations as an important factor in the etiology of milk fever has suggested that the high K or Na content of the diet is more important than the Ca content of the forages in predisposing cows to milk fever (Oetzel 1991). In

this experiment, cows fed the high DCAD had a higher incidence of hypocalcemia compared to cows fed the low DCAD diet in transition period, which may be relative to a higher Na, a low Cl and S content of diet (Table 1), corroborating the other observations (Jonsson 1978; Kurosaki et al. 2007). A major physiologic effect of the additional dietary Na or K was an increase in alkalinity of the blood and urine, because both Na and K are absorbed with nearly 100% efficiency from the diet (Goff 2006; Lean et al. 2006).There is growing evidence that suggests that diets with a highly positive cation-anion difference cause a state of metabolic alkalosis in cows, reducing the responsiveness of bone and kidney to PTH (Gaynor et al. 1989; Goff 1997, 2000, 2008). Therefore, the higher DCAD due to high Na or K content is, the more incidence of hypocalcemia in transition period is.

PTH is the major factor that controls bone osteoclast activity and renal production of DHVD is also under the control of PTH (Horst et al. 1997; Penner et al. 2008). Blood PTH concentrations were highest in the cows fed the alkalinizing diets, cows fed the high K or high Na diets should have had greater concentrations of plasma hydroxyproline at calving than cows fed the diet that was low in K or Na (Goff 1997; Heron et al. 2009). Because concentrations of plasma PTH at calving were higher in cows fed the high CAD diets, the concentration of plasma DHVD and HYP might be expected to be higher in cows fed these diets. In Table 2, compared to other farms, cows in farm II had a higher concentration of plasma PTH (Figure 4), a slight high concentration of plasma DHVD (Figure 5), but a lower concentrations of plasma Ca (Figure 1) and hydroxyproline (Figure 3) during transition period than cows in other farms, suggesting that the bones of the cows consuming the high CAD diets were refractory to PTH stimulation, which has been a consistent finding in most studies of the mode of action of high cation diets (Block 1984; Goff 1991).

Cows fed a high DCAD in transition period, especially around calving, cannot meet Ca demand for maintenance, fetal skeletal development, and lactation from the diet. As a result, these cows undergo a negative Ca balance, which stimulates secretion of PTH in transition period (Jorgensen 1974; Jonsson 1978). The secretion of PTH activates bone osteoclasts, stimulating bone Ca resorption, and activates renal tubules to resorb urinary Ca and to begin producing DHVD in transition period (Goff 1991; Horst et al. 1994). However, these Ca homeostatic mechanisms are active to prevent a severe decline in plasma Ca concentration in the periparturient cows, but the high DCAD may interfere with Ca homeostatic mechanisms (Roche et al. 2003; Liesegang et al. 2007; Taylor et al. 2008).

Based on these experimental observations, it was routinely recommended that dietary strong ion be kept as low as possible in transition period, especially prepartum diet. Despite the fact that it was difficult to limit dietary K or Na content, this strategy was often a successful means to prevent milk fever(Gaynor et al.1989; Horst et al. 1997). To achieve these reduced K or Na diets required that high K or Na forages, such as alfalfa, be removed from the ration and replaced with low Kor Na forages, such ascorn silage or grasshays (Goff 2006; Lean et al.2006; Penner et al. 2008).

These data demonstrate that the most constructive step that can be taken to prevent milk fever or hypocalcemia is to reduce the dietary strong ion (K or Na) content in transition period, especially the prepartum diet.

CONCLUSION

It appears that hypocalcemia was a severe problem in three dairy farms of Heilongjiang province in China, especially a high DCAD farm during transition period. The increase of plasma PTH in the cows fed a high DCAD did not result in an increase in plasma Ca concentration. The lack of response in plasma Ca suggests that bone was not response to PTH, perhaps due to inhibition of high DCAD. This theory is corroborated by plasma HYP concentrations tending to be lower in the cows fed a high DCAD compared to cows fed a relatively low DCAD. These data demonstrated that a high DCAD is a major risk factor for milk fever or hypocalcemia and that the high DCAD may induce metabolic alkalosis in the prepartum dairy cow, which reduces the ability of the cow to maintain Cahomeostasis. However, it is more important for us to need further research in effect of acid-base balance or cation salts on milk fever or hypocalcemia in future.

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Chapter 7

HAEMODYNAMIC CHANGES OF THE SUPER-OVULATED CORPUS LUTEUM IN CATTLE

Taymour M. EL-Sherry[1], Motozumi Matsui[2] and Yoh-Ichi Miyake[2]

[1]Department of Theriogenology, Assiut University, Assiut, Egypt,
[2]Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555, Japan

ABSTRACT

The aim of this study was to explore the real time changes in the vascularity of growing super-ovulated corpus luteum and to compare it with the cyclic CL. Eighteen Holstein-Friesian cows were classified into 2 groups. Group 1; cyclic CL (CCL), in which 7 animal were left to get naturally ovulated while group 2; super-ovulated CL (SCL) (n=11) received PGF2α after 10 days of spontaneous ovulation. After 36 hours, all follicles larger than 5 mm were aspirated at day 0 (D0). The animals were given 28 A.U FSH 24h after aspiration and for 4 days (twice daily, 12h interval). On day 5, the animals received the GnRH analogue. The growth of the CL was examined by Doppler ultrasonography to detect the changes in the blood vasculature in different stages. Blood samples were collected on a daily basis and were used to detect Progesterone (P4) using enzyme immune assay (EIA). The results showed that the super-ovulated CL was significantly smaller than the cyclic CL but the blood area percentage were significantly ($P<0.05$) higher in (SCL) than the (CCL). It

also showed that the P4 level was associated with the vascular activity rather than the size of the Cl.

In conclusion, the super-ovulated CL has a smaller size, shorter lifespan and less P4 production although the high activity of the blood vasculature was higher in comparison to the cyclic CL.

Keywords: Corpus luteum, Doppler, Progesterone, superovulation

INTRODUCTION

The ovarian corpus luteum plays a critical role in reproduction because it is the primary source of circulating progesterone (P_4). After ovulation, the wall of the ruptured follicle starts to grow and vascularize rapidly. In fact, the rate of tissue growth and angiogenesis of the CL rival those of fastest growing tumors. The extent of angiogenesis within the CL reaches a maximum within 2-3 days after ovulation (Reynolds *et al.*, 2000). As the CL matures, the majority of the steriodogenic cells establish content with one or more capillaries (Reynolds *et al.*, 1992; Grazul-Bilska *et al.*, 1992). Thus, making the CL one of the most highly vascularized organs of the body (Gaytan *et al.*, 1999). Indeed, 50% of the bovine mature CL cell account is vascular endothelial cells (O'Shea *et al.*, 1989).

In the super-ovulation, the progesterone level plays a critical role in the embryo yield and quality. In fact, 16 % of the super-ovulation variability was due to the progesterone level (Kweon *et al.*, 1986). The irregular progesterone profile in donors correlate negatively with the super-ovulation response and embryo quality (Callesen *et al.*, 1988; Wubishet *et al.*, 1991). The low plasma progesterone concentration and the delay in the rise of progesterone after ovulation were associated with retarded embryo growth and low secretion of interferon –tau ($IFN_{-\tau}$) (Mann and Lamming, 2001).

The color-flow Doppler ultrasonography was used as a non-invasive tool for the evaluation of the CL blood flow during its development (Acosta *et al.*, 2003; Acosta *et al.*, 2002) and luteolysis in cattle(Miyamoto *et al.*, 2005). The blood flow of early CL gradually increased in concomitant with the growth of the CL volume and progesterone concentration from D2 to D5 (Acosta *et al.*, 2003; Acosta *et al.*, 2002), while with spontaneous luteolysis, the blood flow acutely increased on D17-18 followed by a decrease in plasma progesterone concentration one day later (Miyamoto *et al.*, 2005).

The aim of this study is to focus on the changes of local blood flow and progesterone level of the super-ovulated CL and to compare it with the cyclic CL.

MATERIALS AND METHODS

Eighteen non-pregnant (Holstein-Friesian), four to five year old cows and of a body weight ranging from 400 to 500 kilograms were kept under a normal management program at the Animal Science and Agriculture Farm, Obihiro University, Japan. From the gynecological point of view, all animals were of normal health, reproductive soundness, and were exhibiting normal estrous cycles.

Experimental Design

Eighteen Holstein-Friesian cows were classified into 2 groups. Group 1; cyclic CL (CCL)(n=7) animals were synchronized with double injection of $PGF_{2\alpha}$(Estrumate, 5ml, Sumitomo pharm. Co., Osaka, Japan) and left to get naturally ovulated. Group 2; super-ovulated CL (SCL) (n=11) animals received $PGF_{2\alpha}$ after 10 days of spontaneous ovulation. After 36 hours, all follicles($\geq$ 5mm) were aspirated by transvaginal ultrasound-guided follicle aspiration. For the ultrasound guidance of the aspiration needle, an ultrasound scanner (SSD-5500, ALOKA CO., Ltd., Tokyo, Japan) was used and equipped with a 7.5 MHz transvaginal convex transducer (UST-M15-21079, ALOKA CO., Ltd.) with an 18-gauge single-lumen attached stainless steel needle guide. The animals were given 5, 4, 3, 2 Armour units (A.U.) of porcine FSH (ANTRIN R 10, Kawasaki Pharm. Co., Kawasaki Japan) twice daily at 12 hour intervals for four consecutive days, respectively. The animals received the GnRH analogue (Fertirelin acetate 100μg; Conceral; Nagase Pharm. Co., Osaka, Japan) 12h after the last dose of FSH.

Monitoring of Corpus Luteum Development

The growing CLs were examined by transrectal ultrasonography. All scans were performed by the same investigator. Each CL was measured at its maximum diameter. After morphological evaluation, the power flow mode of

the ultrasound scanner was activated for blood flow mapping. Color signals were used to evaluate the blood flow around the entire perimeter of the CL. The sectional area (SA) of the CL was estimated by the following equation $SA= \pi/4x\ (SD)^2$, where SD is the sectional diameter (Acosta *et al.*, 2003). The colored area in the image that was obtained at the maximum diameter of the CL was used as quantitative index to express the blood flow within the CL wall. Areas of color represent regions with a flow velocity higher than 2 cm^{-1}. Scan-recorded images were stored on a Magneto optical (MO) disk drive (Maxoplix Corporation). The CL sectional area (SA), the blood flow area (BFA) and blood flow area percent (BFA%) which estimated as *BFA%= BFA/SA x 100* were quantified using Image J program (version 1.62) developed at the USA National Instituted of Health (http://rsb.inf.nih.gov/ij). The changes in the SCLs were profiled using a retrospective evaluation of ovarian sketches that provided topographical, dimensional and colored area. To overcome the problem of tracking a large number of super-ovulated CLs, a sectional method of sketching CLs was used in which multiple ovarian maps were made for each ovary, while moving the transducer from medial to lateral aspect of the ovary (Jaiswal *et al.*, 2004). The blood flow velocity wave forms were recorded during three cardiac cycles to determine the time averaged maximum velocity (TAMXV) for the SCLs. It was difficult to records the TAMXV for the same SCL daily so TAMXV of 2 to 3 different SCLs (n=3 cows) at the same day was estimated and expressed in Mean ± SEM.

Blood Collection and Hormonal Determination

The blood samples were collected after ovulation and every 24-h until spontaneous luteolysis by caudal venipuncture using 10 ml heparinized tube. The concentration of P_4 was determined by double-antibody enzyme immunoassays (EIA) (Miyamoto *et al.*, 1992). The recovery rate was 87%. The standard curve ranged from 0.05 to 50ng/ml, and the ED_{50} (effective dose 50) of the assay was 7.3ng/ml. Intra- and interassay coefficients of variations (CVs) were 2.9 and 9.3%, respectively.

Statistical Analysis

The day of ovulation was considered as (=D0). The data of hormonal concentration, CL sectional area (SA), blood flow area (BFA) and blood area

percent (BFA%) were expressed as mean ± SEM. All data was analyzed by repeated measures of analysis of variance (ANOVA) to determine the main effects of thegroup and interaction of the group by day. When the main effect of the group or group by day was observed, the difference of group means at specific time point were analyzed by the Student's ***t***-test using JMP statistical software (version 5.1; SAS Institute, Cary, NC, USA). The different means were significant at $P<0.05$.

RESULTS

Ovarian Response to the Super-ovulation Protocol

According to the super-ovulation response, the animals classified to 2 subgroups. Good response (n=5) 8-15 SCLs with a mean (11.25±1.5) and Poor response (n=6) 1-5 SCLs with a mean of (2.8±0.8).

Morphological Changes of the Haemodynamics of the Super-ovulated CL:

In the early CCL and SCLs, the blood flow area gradually increased in concomitant with the increase in the CL volume. The color signals are primary at the periphery and outer portion of the both CCL and SCL (Figure 1 A,B). The signals started at the base of the newly-formed CL to the opposite apex (Figure 1 C,D). The color signals were completely surrounding the SCLs at D5 while they start to surround the CCL at D7 (Fig 1 E-H). The SCLs were supplied by a branch of blood vessel which gave small branches for each SCL, making a network of blood vessels (Figure 2).

The blood flow surrounded the CL at spontaneous luteolysis increased at D16 for the CCL and at D12 for the SCLs (Figure 3).

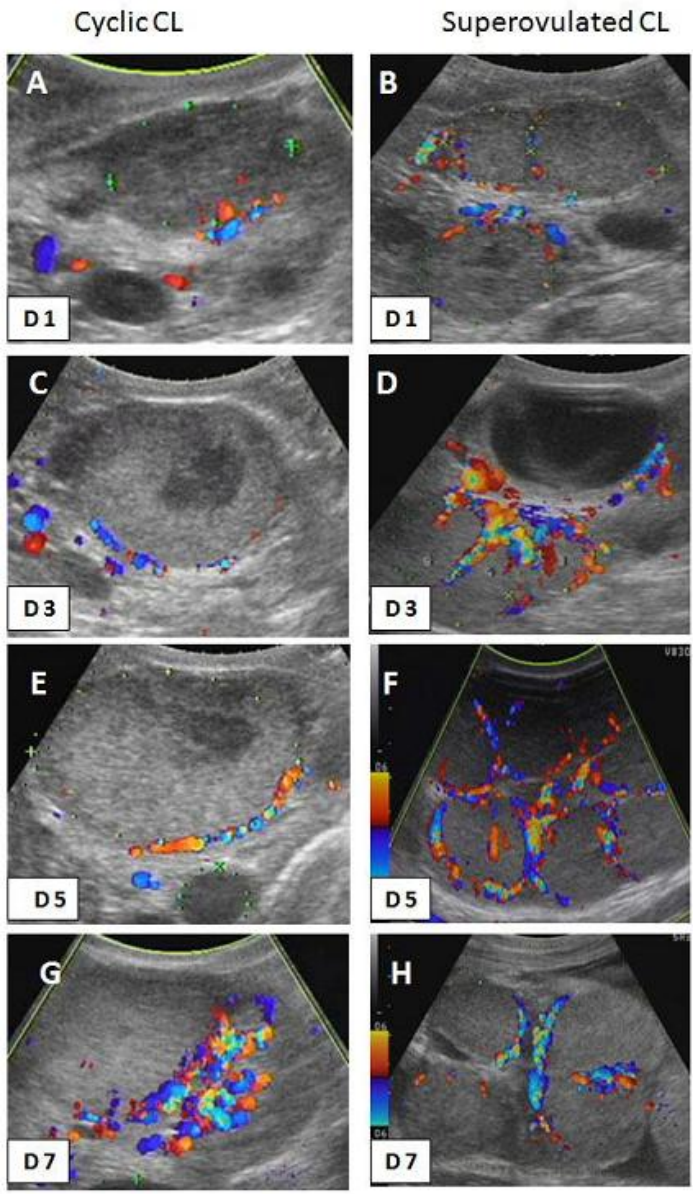


Figure 1. Representative color Doppler image of the cyclic and super-ovulated CLs. These images shows real time changes of the developing luteal blood flow area. A - B) Luteal Blood flow area appear at the base of the CL after ovulation. C-D) the color signals started at the periphery and outer portion of the CL. E-F) The color signals completely surround the outer layer of the SCLs while partially surround the CCL.

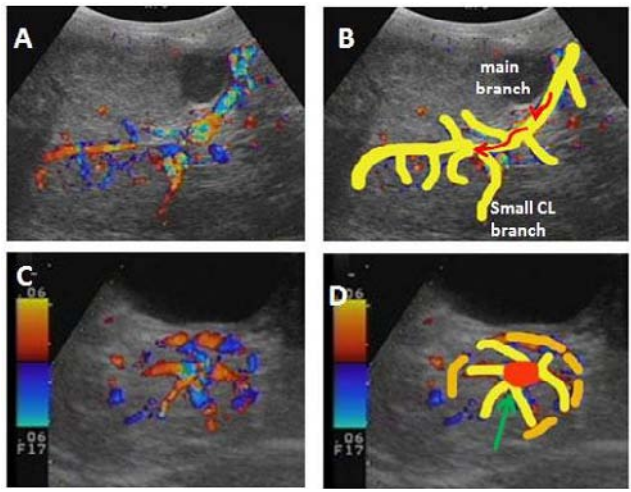


Figure 2. A-B) A branch of blood vessel which gave small branches for each SCL making a network of blood vessels. C-D) Image of a single SCL with base point at which the small branch starts to surround the periphery of the CL.

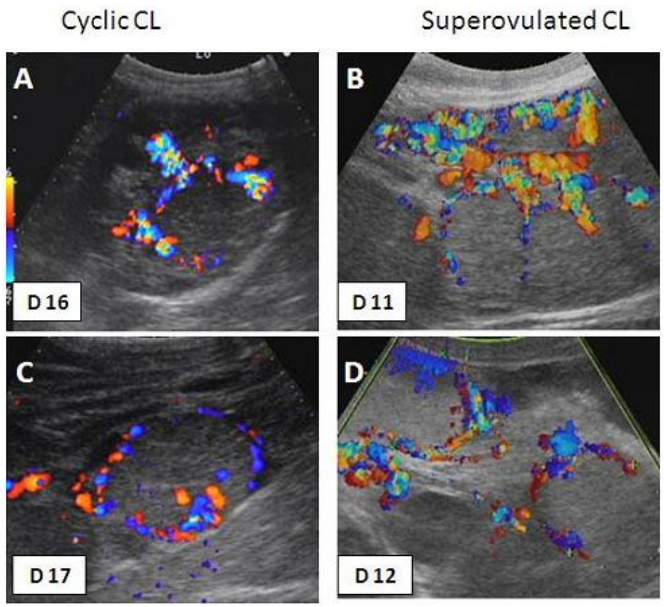


Figure 3 Representative color Doppler image if the cyclic and super-ovulated CLs. These images shows real time changes of spontaneous luteolysis of blood flow area. A-B) Increase luteal blood flow area surrounding CL during spontaneous luteolysis on D16 for CCL and D11 for the SCLs. C-D) Decrease of the luteal blood flow next day.

CL Area, Blood Flow Area, Blood Flow Area % and TAMXV

Between the groups, the results showed that there was no different between the size of the CCL and SCLs for the first 3 days of development. At day 5, the size of the CCL was significantly ($P<0.05$) increased and became larger than that of the SCLs (Figure 4a).

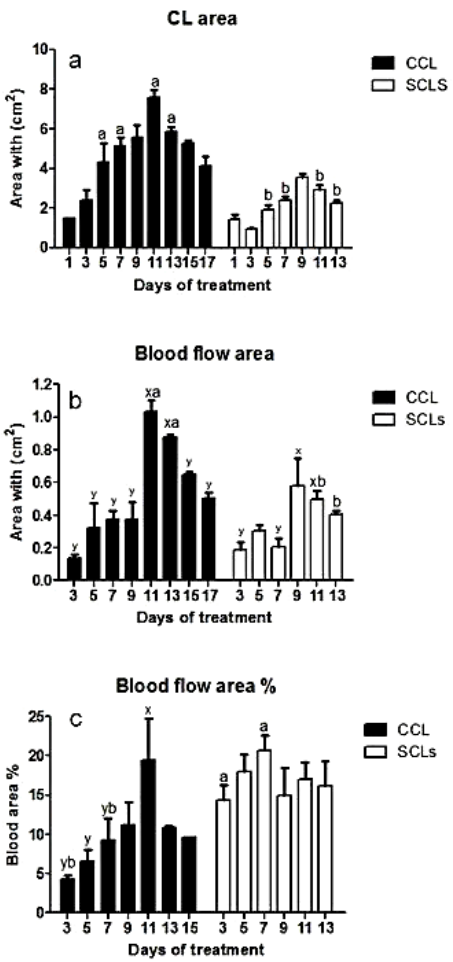


Figure 4 Comparative changes in a) CL area, b) blood flow area (BFA) and c) blood flow area % (BFA%) between cyclic CL (CCL)and super-ovulated CL (SCLs). Value are mean ±SEM of each time period. ^{a,b} significant at $P<0.05$ between groups, ^{x,y} Significant at $P<0.05$ within the same group.

The blood flow area for both CCL and SCLs were similar until D11. After that, the BFA of the CCL increased significantly (P<0.05) from D11 to D13 in comparison to the SCLs (Figure 4b). On the contrary, the blood flow area percent (BFA%) of the SCLs was significantly (P<0.05) larger than that of CCL until D7. The results showed that BFA% of SCLs was almost triple that of the CCLs at D3 (14.4±2 vs. 4.3±5) and double at D7 (20.7±2 vs. 9.3±2.8) (Figure 4c).

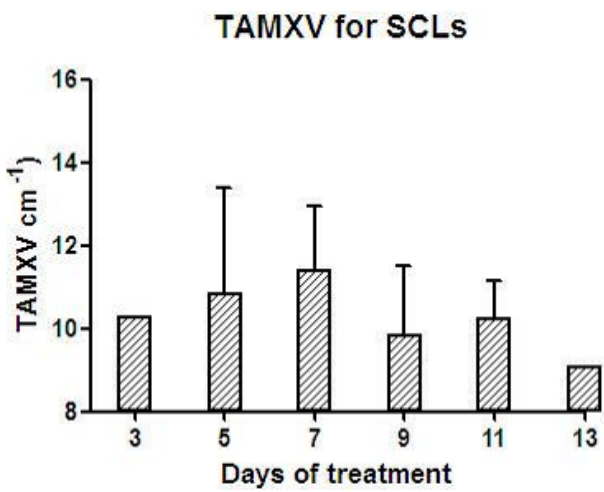


Figure 5. Changes in a) time-averaged maximum velocity (TAMXV) of the SCLs. Data points show mean ±SEM of each time period (n= 3 cows).

Within the same groups, it was found that the blood flow area increased significantly (P<0.05) at D11 (1.03±0.07 cm^2) for the CCL while increased significantly (P<0.05) at D9 (0.58±0.17 cm^2) for the SCLs. It was found that the blood flow area % of the CCL increased significantly at D11 (19.5±5.4%) while the blood flow area % for the SCLs was not different all over its lifespan. The TAMXV for the SCLs was estimated. The results showed that the TAMXV was associated with the (BFA%) and P4 concentration (Figure 5).

Progesterone

The analysis of P4 from D1 to D7 revealed an effect of group (P<0.05) and an effect of group by day (P<0.05) (Figure 6). Two patterns of super-

ovulation P4 level have been estimated according to the number of the CL of each sub-group. The P4 level of the 2 sub-groups was similar for the first 3 days. After that, the P4 of the Good-subgroup increased rabidly and significantly ($P<0.05$) at D3 and reach the maximum and plateau level (10.4±2.5 ng/ml) at D6. Similarly, the P4 of Poor sub-group reach plateau at D6 but the level did not exceed (3±0.4ng/ml). In the CCL, the P4 level gradually increased at D3 to reach the plateau at D11 and maximum at D14 (10.3±0.9 ng/ml). Spontaneous luteolysis started at D13 for SCLs while started at D19 for CCL. The results showed that there is no significant different in the maximum concentration of P4 produced by CCL and Good sub-group SCLs.

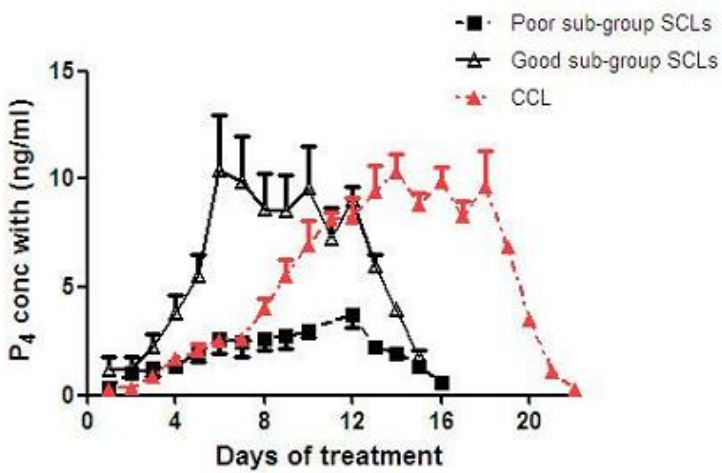


Figure 6. P4 level of the CCL and SCLs sub-groups. Values are mean ±SEM of each time period.

DISCUSSION

Using of the Doppler ultrasonography was important for evaluating the functional status of the cyclic and super-ovulated CL. Several reports indicated that the ultrasonography assessment of CL size was correlated with milk and plasma progesterone concentration and was a viable alternative to the measurement of plasma progesterone for the evaluation of luteal function (sprecher et al 1989; Kastelic et al 1990; Assey et al 1993 Ribadu et al 1994). However, this correlation was absent during luteal regression. The rate of the progesterone regression was faster than the rate at which the diameter of the

CL decreased (Kastelic, 1990, assey et al 1993). A positive correlation between the CL blood flow area, CL volume and plasma progesterone level was proved by (Miyazaki et al 1998).

The results showed that the size of the CCL was significantly ($P<0.05$) larger than the size of the SCLs. It was reported that the weight and volume of the super-ovulated CL was 50% smaller than that of the cyclic CL in heifers at D7 (Maciel *et al.*, 1992). Similarly, the ovulation of smaller follicles produced 53% smaller CL at D7 and 71% at D14 which lowered fertility and decreased circulating progesterone concentration (Vasconcelos *et al.*, 2001). Supporting that observation, a study of the CL size and function following single and double ovulation in non-lactating dairy cows revealed that the double ovulation produce smaller corpora lutea (Mann *et al.*, 2007).

The SCLs showed active blood flow activity during the first 7 days of its development in comparison to the CCL. Such activity demonstrated by the increase in the (BFA), (BFA%) and TAMXV. Many authors studied the histological difference between the SCLs and the CCL (Maciel *et al.*, 1992; Vaughan *et al.*, 1996). They found no morphological difference between the luteal tissue of the SCL and CCL of heifers at day 7 (Maciel *et al.*, 1992) except that the SCLs had more connective tissue and smaller in volume than cyclic CL (Vaughan *et al.*, 1996). In fact, those studies were performed on the SCLs and CCL at D7, not during the early developmental stage. A possible explanation of blood flow hyperactivity of SCLs may be associated with the number of blood clots formed during super-ovulation. Those blood clots might act as active stimuli for endothelial cell migration (Robinson *et al.*, 2009) and may be more effective than one clot formed by spontaneous ovulation. Indeed, the platelets of the blood clot were potent stimuli for endothelial cells migration (Furukawa *et al.*, 2007).

In the present study, the SCLs exhibited a compressed (shorter) lifespan lasting 12 days. This SCLs were similar to the short-lived CL described in the first ovulatory estrus in prepuberal heifers (Berardinelli *et al.*, 1979) and post-partum cows (Corah *et al.*, 1974). An explanation of this phenomenon might be the increased proportion of the large luteal cells which have high affinity receptors to PGF2α (Niswender *et al.*, 1985). Other researchers have presented evidence in vivo (Rajamahendran and Calder, 1993) and in vitro (Rusbridge *et al.*, 1992) suggesting that GnRH-induced CL have a luteolytic mechanism capable of responding to luteolysin when spontaneous-derived CL of similar age do not respond.

The blood flow temporary increased before the spontaneous luteolysis of the SCLs and CCL then followed by a decrease in progesterone one day later.

The same observation was reported by (Shirasuna *et al.*, 2004) who found that the BFA increased on D17-18 in the cow, as one of the main factors triggering the luteolytic cascade in the cow.

The P4 profile of both SCLs sub-groups showed that the SCLs were sub-functional and produce less P4 than spontaneous CL of similar age. The P4 level of the Good sub-group (8-15 CL) was nearly equal to the P4 level produced by a single CCL. Likewise, hCG-induced CL (Sianangama and Rajamahendran, 1996) and the GnRH-induced CL (Vasconcelos *et al.*, 2001) are small in size and secrete low P4. Such subnormal function could be attributed to an inadequate development of the super-ovulatory follicles.

The P4 level for the SCLs increased rapidly to reach the maximum level within 6 days after ovulation in contrary to the P4 level of the CCL which take a longer time. Such a P4 pattern was associated with the increase of the BFA, BFA% and TAMXV of the SCLs. This P4 pattern may be important for embryo quality produced by super-ovulation. Several reports found that the low P4 concentration and delay in the rise of P4 after ovulation were associated with retarded embryo growth, low secretion of interferon tau (IFN-t) and lead to luteal regression (Mann and Lamming, 2001).Moreover, the quality of the embryo increased with increasing the level of P4 at D5 after {please complete sentence}(Wubishet *et al.*, 1991; Yadav *et al.*, 1986).

CONCLUSION

In conclusion, the super-ovulated CL has a smaller size, shorter lifespan and less P4 production although the high activity of the blood vasculature was higher in comparison to the cyclic CL.

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